**A**

**Summary Report**

**on**

***'Screening of Medicinal Plants from Genus Terminalia for Treatment of Type 2 Diabetes Mellitus'***

**To be submitted**

**to**

**Research and Recognition Committee**

**of**

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## 1. Introduction:

Type 2 diabetes (T2DM) is a serious global health problem that accounts for 80 % to 95 % of all reported diabetes cases. Alarmingly the global prevalence of diabetes has reached 425 million people. There will be 629 million people with diabetes in the World in 2045. (IDF)

Overnutrition, reduced physical activity, epigenetics, genetics and environmental factors play a crucial role in the prevalence of type 2 diabetes (Paneni et al.2013, Yacoub et al.2014). T2DM is characterised by chronic irregular metabolism of carbohydrates and lipids. It causes functional and structural changes to the vascular system and ultimately leads to the development of diabetes-related microvascular complications in the eye, kidney and peripheral nerves and atherosclerotic macrovascular complications affecting arteries of the heart, brain and lower extremities (Paul et al.2001, Mortuza et al. 2014). The risk of development of cardiovascular disease is two to four times more in people with T2DM compared with non-diabetics. Amputation in the lower extremity is common in people suffering from T2DM. Additionally, T2DM is the leading cause of end-stage renal failure, visual impairment and blindness in both developed and developing countries (Kume et al., 2010). Thus, T2DM and the vascular complications associated with it are now considered to be a major health issue worldwide, and new therapeutic approaches are needed to treat T2DM and associated vascular complications.

**Pathophysiology of type I Diabetes:**

The autoimmune destruction of pancreatic β-cells, leads to a deficiency of insulin secretion which results in the metabolic derangements associated with IDDM. In addition to the loss of insulin secretion, the function of pancreatic α-cells is also abnormal and there is excessive secretion of glucagon in IDDM patients. Normally, hyperglycemia leads to reduced glucagon secretion; however, in patients with IDDM, glucagon secretion is not suppressed by hyperglycemia. Deficiency in insulin leads to uncontrolled lipolysis and elevated levels of free fatty acids in the plasma, which suppresses glucose metabolism in peripheral tissues such as skeletal muscle. This impairs glucose utilization and insulin deficiency also decreases the expression of a number of genes necessary for target tissues to respond normally to insulin such as glucokinase in liver and the GLUT 4 class of glucose transporters in adipose tissue (Ozougwu 2013).

**Pathophysiology of type 2 diabetes (NIDDM)**

On the basis of oral glucose tolerance testing the essential elements of NIDDM can be divided into four distinct groups:

i) Those with normal glucose tolerance.
ii) Chemical diabetes (called impaired glucose tolerance).
iii) Diabetes with minimal fasting hyperglycemia (fasting plasma glucose less than 140 mg/dl).
iv) Diabetes mellitus in association with overt fasting hyperglycemia (fasting plasma glucose greater than 140 mg/dl).

The individuals with impaired glucose tolerance have hyperglycemia inspite of having highest levels of plasma insulin, indicating that they are resistant to the action of insulin. In the progression from impaired glucose tolerance to diabetes mellitus, the level of insulin declines indicating that patients with NIDDM have decreased insulin secretion. Insulin resistance and insulin deficiency are common in the average NIDDM patients (Holt, 2004). Insulin resistance is the primary cause of NIDDM, however some researcher contend that insulin deficiency is the primary cause because a moderate degree of insulin resistance is not sufficient to cause NIDDM. Most patients with the common form of NIDDM have both defects. Recent evidence has demonstrated a role for a member of the nuclear hormone receptor super family of proteins in the etiology of type 2 diabetes. Relatively new classes of drugs used to increase the sensitivity of the body to insulin are the thiazolidinedione drugs. These compounds bind to and alter the function of the peroxisome proliferators-activated receptor g (PPARg). PPARg is also a transcription factor and when activated, binds to another transcription factor known as the retinoid x receptor (RXR). When these two proteins are complexed a specific set of genes becomes activated. PPARg is a key regulator of adipocyte differentiation; it can induce the differentiation of fibroblasts or other undifferentiated cells into mature fat cells. PPARg is also involved in the synthesis of biologically active compounds from vascular endothelial cells and immune cells (Ozougwu 2013).

**Challenges in diabetes drug development:**

The TZD's entered the world market with the blockbuster prototype Troglitazone. The class was considered unique because of its' novel mechanism of action and its ability to address the problem of insulin resistance. In spite of an extensive development program Troglitazone lasted in the world

market for only a few years before the association of Troglitazone use and severe liver disease sometimes resulting in death was determined. The frequency of severe liver disease with Troglitazone use was identified as 1 in 10,000 and therefore not identified during routine clinical development. Rosiglitazone faced the same fate only several years after its launch globally due to the occurrence of increased cardiovascular events with its use and was associated with a significantly increased risk of heart attack (odds ratio =1.43, (95% confidence interval, 1.03 to 1.98; $P = 0.03$)), and an even higher risk of death from all cardiovascular diseases (odds ratio = 1.64) eventually leading to the withdrawal of Rosiglitazone from the world market. The association of Pioglitazone and bladder cancer has probably put the "nail in the coffin" for this entire therapeutic class of drugs.

## 2. Statement of Problem:

### Diabetes drug discovery and development:

India has and will continue to be a major contributor to the overall burden of diabetes globally. The diabetes epidemic can be curtailed and possibly reversed if we incorporate a culture of healthier eating and regular exercise in our society. For subjects with diabetes mellitus optimal use of existing therapeutic options and the search for newer more effective and safer therapies will go a long way in reducing the complications of diabetes there by reducing morbidity and overall mortality (Vaz and Patnaik 2012).

### Overview of current synthetic drugs in the treatment of T2DM:

### Biguanides:

The class of biguanides includes the metformin and two withdrawn agents phenformin and buformin. The reason for removing phenformin and buformin from the market was the occurrence of fatal lactic acidosis Introduced in the market in 1950; metformin is a well-accepted first-line choice for the treatment of T2DM due to its good efficacy, low price and low rate of adverse effects especially in long-term use. Metformin reduces fasting plasma glucose concentrations by reducing rates of hepatic glucose production through a reduction in gluconeogenesis and glycogenolysis. Metformin also affects peripherally and improves skeletal myocyte glucose uptake, reduces the overall plasma free fatty acid (FFA) concentration and induces mild weight loss through reduction of caloric intake.

**Sulphonylureas**

These drugs specially stimulate insulin secretion in aglucose-independent manner by binding to a regulatory protein called Sulphonylureas receptor on the pancreatic b-cells. After binding to the receptors, they trigger closure to KATP channels, membrane depolarization and influx of calcium through voltage-dependent channels, which subsequently ends up in insulin secretion. In addition to their pancreatic effects, sulphonylureas can enhance insulin stimulated peripheral glucose utilisation through triggering insulin action on adipose tissue glucose transport and lipogenesis and skeletal muscle glycogen synthase.

**Thiazolidinediones:**

Troglitazone was subsequently withdrawn from use, in March 2000, because of causing severe hepatic toxicity. Two currently available PPAR-g agonists, Rosiglitazone and pioglitazone, were approved in 1999. This class of agents also known as glitazones like the biguanide metformin do not increase insulin secretion but rather increase insulin sensitivity in muscle and adipose tissue and in the liver by activating PPAR-g and affecting gene regulation in the target cells.

The potential of thiazolidinediones to lower glucose production by the liver or improve glucose transport into muscle and adipose tissue have been shown. The main adverse events associated with the thiazolidinediones class are weight gain and fluid retention that may be severe enough to exacerbate or precipitate heart failure. These drugs also cause light decrease in the haemoglobin level and haematocrit, probably without clinical consequence. Pioglitazone and rosiglitazone are licensed for use as the second-line therapy to metformin and glyburide, agents that have demonstrated efficacy in decreasing the microvascular and macrovascular complications associated with T2DMDisaccharidase inhibitors (a-glucosidase inhibitors) Glucagon-like peptide 1 agonists DPP-4 inhibitors Sodium-glucose transporter-2 inhibitors Bile acid sequestrants /dopamine-2 agonists (Safavi, Foroumadi, and Abdollahi 2013).

**Diabetes complications and therapeutic targets:**

The normal rate of hepatic glucose production increases in T2D patients, resulting in decreased insulin secretion (due to reduced number of β cells), increased glucagon secretion and hepatic insensitivity to insulin. Collective effect of all these phenomenon's damages the healthy glucose uptake of adjacent tissues. Several factors regulate receptor mediated insulin resistance mechanism. Few of the significant factors include elevated serine/ threonine phosphorylation of

the insulin receptor, deactivation of the receptor, decreased tyrosine phosphorylation, autoantibody formation against the receptor and inherited structural abnormality of the receptor. Disruption of insulin action also arises at post-receptor events, chiefly glucose transport. There are a number of different hypotheses explaining the mechanism of insulin resistance in obesity. It was proposed that tumour necrosis factor alpha (TNF-α) is released by adipose tissue and is able to impair insulin signalling through serine kinase and tyrosine phosphatase dependent modulation of the insulin signalling chain at the level of the insulin receptor and substrates (IRS) (Banu and Bhowmick 2017).

**Herbal Therapy for T2DM:**

Long before the birth of orthodox Western medicine, medicinal herbs were applied to treat a wide range of disease categories. Due to emphasis on scientism and other complicated reasons, Western medicine now prevails over "traditional" forms of medicine including herbal medicine systems.

Medicinal herbs have never become obsolete and still play a prominent role in human health care. Among them, over1200 plants have been claimed to be remedies for diabetes. Over 400 plants as well as 700 recipes and compounds have been scientifically evaluated for T2D treatment. Metformin was developed based on a biguanide compound from the antidiabetic herb, French lilac, and is now a first line drug for T2D.

Medicinal herbs contain diverse bioactive compounds and can have multiple actions on insulin action, insulin production, or both. In the present review, we focus on scientific studies of selected glucose lowering herbs and phytocompounds and their ability to target insulin resistance, $\beta$-cell function, incretin-related pathways, and glucose (re)absorption. Phytochemistry, antidiabetic bioactivities, and likely modes of action of the selected plants and compounds are discussed (Chang et al. 2013).

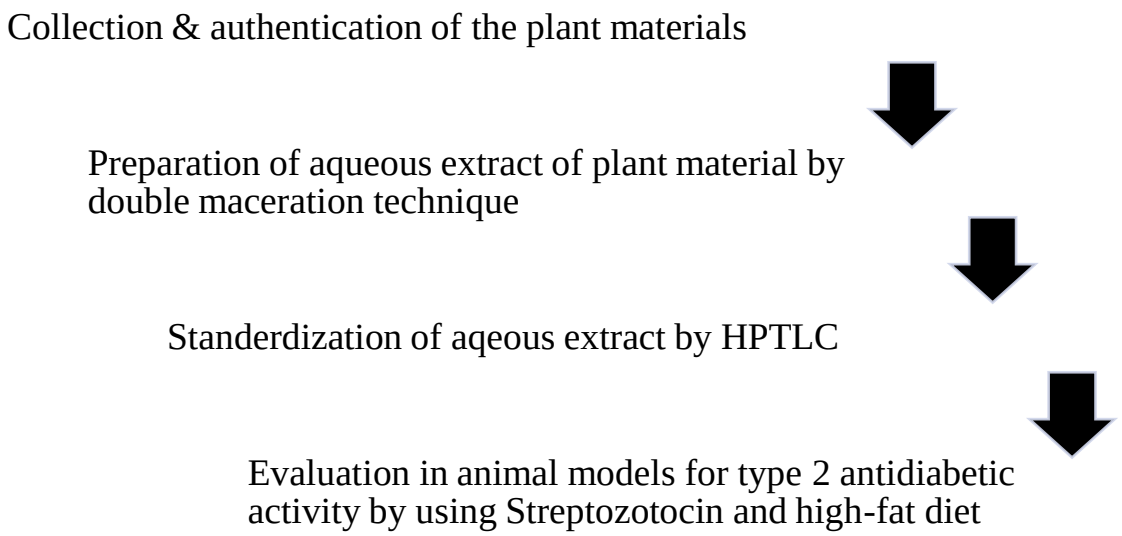
## 3. Rationale of the study

1. Preliminary study was carried on these medicinal plants for T1DM only.
2. The selected plants contain Phenolic compounds which have proven antidiabetic effects.
3. These plants also reported in traditional literature.

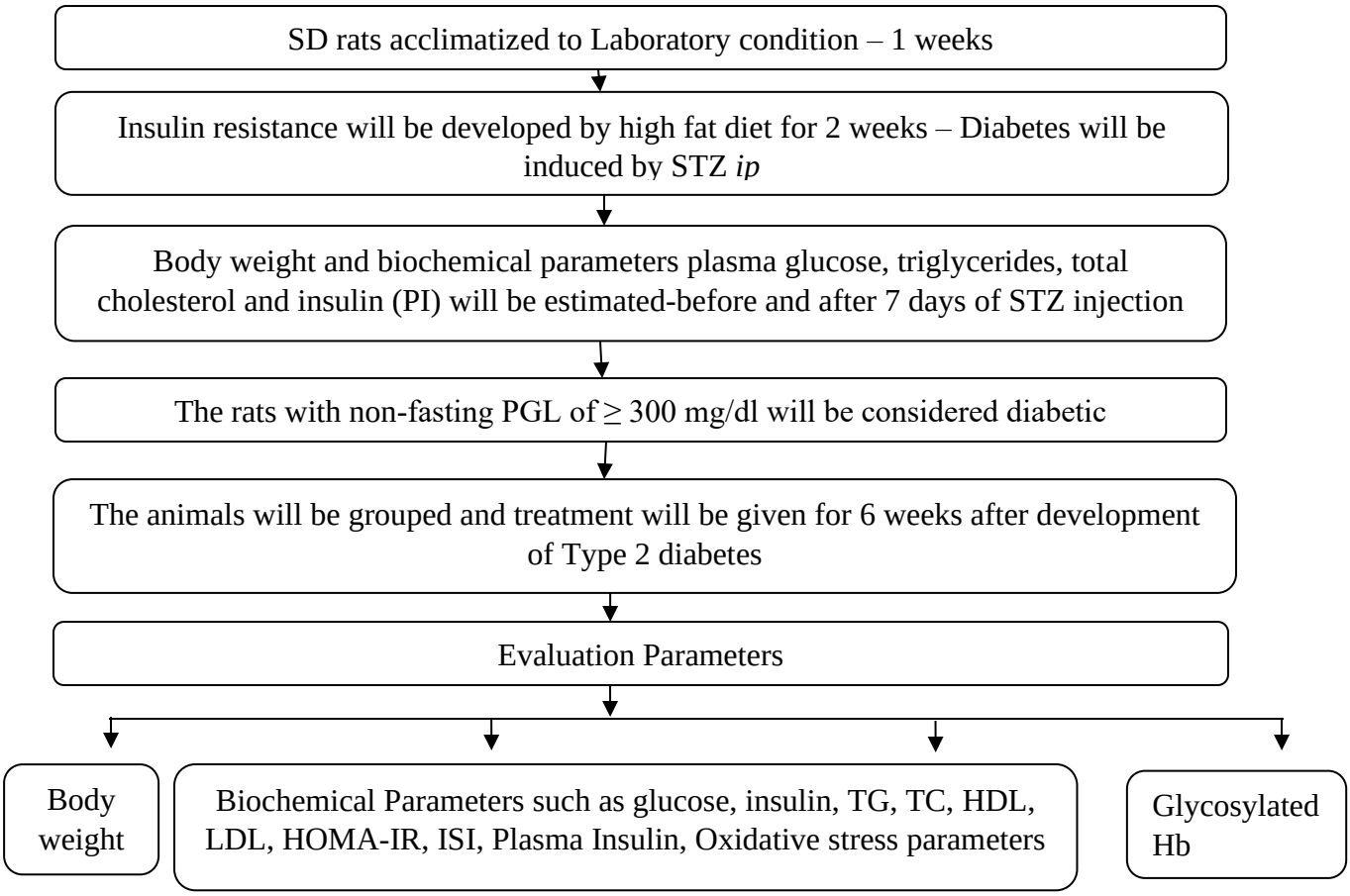
## 4. Objectives:

1. To prepare and standardize extracts of *Terminalia bellirica, Terminalia chebula, Terminalia catappa.*
2. To study the effect of *Terminalia bellirica* extract in experimental model of Type 2 Diabetes Mellitus.
3. To study the effect of *Terminalia chebula* extract in experimental model of Type 2 Diabetes Mellitus.
4. To study the effect of *Terminalia catappa* extract in experimental model of Type 2 Diabetes Mellitus.

## 5. Work Plan


Collection & authentication of the plant materials
Preparation of aqueous extract of plant material by double maceration technique
Standerdization of aqeous extract by HPTLC
Evaluation in animal models for type 2 antidiabetic activity by using Streptozotocin and high-fat diet

**Methodology:**


SD rats acclimatized to Laboratory condition – 1 weeks
Insulin resistance will be developed by high fat diet for 2 weeks – Diabetes will be induced by STZ *ip*
Body weight and biochemical parameters plasma glucose, triglycerides, total cholesterol and insulin (PI) will be estimated-before and after 7 days of STZ injection
The rats with non-fasting PGL of ≥ 300 mg/dl will be considered diabetic
The animals will be grouped and treatment will be given for 6 weeks after development of Type 2 diabetes
Evaluation Parameters
Body weight
Biochemical Parameters such as glucose, insulin, TG, TC, HDL, LDL, HOMA-IR, ISI, Plasma Insulin, Oxidative stress parameters
Glycosylated Hb

## 6. Work Done

**I Aim:** To study the effect of *Terminalia bellirica* extract on the experimental model of Type 2 Diabetes Mellitus.

### Experimental Section

### Crude drugs and Chemicals

*Terminalia bellirica* fruits are procured from the D.G. Ayurvedic Sangrah Andheri (W), Mumbai, India. The fruits were authenticated by the Scientist, Herbal Drug Administration at Agharkar Research Institute, Pune, MS, India. A voucher copy of dried fruits has been submitted to the research laboratory for further reference (Voucher No. 3/187/2018/adm. 252, 03.12.2018). Casein was purchased from Clarion Casein Limited (Kadi, Gujarat), India. Streptozotocin was purchased from Sigma Aldrich (St. Louis, MO, United States). DL-methionine and Yeast extract powder were procured from Molychem, Mumbai, India. Cholesterol was purchased from Mumbai, India (Loba Chemie Pvt. Ltd.), and Sodium chloride was procured from SD Fine Chemicals, Mumbai, India. Ellagic acid was purchased from Merck and Gallic acid was purchased from Sigma Aldrich. Diagnostic kits were purchased from Transasia Biomedicals Ltd., India. Rat insulin ELISA kit was procured from Abbkine, USA. SIRT1 primary antibody was procured from Santa Cruz Biotech, USA.

### Preparation of Aqueous Extract

Kernels of dried fruits were removed, and powdered kernels were used to prepare aqueous extract (AqE). Powdered kernels (500 g) were extracted using the maceration process in 5000 mL distilled water. The extract was stored in the refrigerator until further use (Kulkarni et al. 2013).

### Standardization of Aqueous Extract of Fruits of *Terminalia bellirica* by HPTLC

The Ellagic acid and gallic acid contents in the prepared aqueous extract of *T. bellirica* were determined by a HPTLC method. The chromatographic analysis was carried out on Camag HPTLC (Muttenz, Switzerland) equipped with visionCATS HPTLC Software, CAMAG Linomat V applicator, CAMAG Hamilton Micro syringe (100 µL), CAMAG TLC Scanner 4 (4.6 mm×25 cm, 5 µm), CAMAG UV Cabinet (254 & 366 nm), CAMAG Twin Trough Chambers

(20 x 10 cm). The separation was performed using solvent system of *Methanol: Water: Formic acid (4:6:0.5) v/v/v.* The stationary phase was Merck, HPTLC plate Silica gel 60 RP-18. The scanning was done at 254 nm.

**Preparation of Standard Solutions**

The standard solutions of Ellagic acid and gallic acid of 100 µg/mL were prepared in methanol and applied into the HPTLC system.

**Preparation of Extract Solution**

To determine the percent content of Ellagic acid and gallic acid present in the aqueous extract of *T. bellirica*, 50 mg of extract was weighed and diluted with 5 ml of methanol. From the resulting solution, 1 mL is diluted to 10 mL of methanol. The prepared solution was used for further analysis. The above-prepared standard and extract solutions were applied into the HPTLC system. The peak area and retention factor of tannins in the extract were compared for standardization of the contents of Ellagic acid and gallic acid in the extract.

**Experimental Animals**

From the National Institute of Biosciences, Pune, MS, India, male *SD* rats (160–170 g) were obtained. The male *SD* rats were housed in the central animal house at relative humidity (75 ± 5%), standard temperature (22 ± 2°C), and a light/dark cycle of twelve hours during the study. The housed rats were given a normal pellet diet obtained from Nutrimix Laboratory Animal Feed, MS, India, along with purified water ad libitum. The Institutional Animal Ethics Committee approved the research protocol-wide number CPCSEA/IAEC/P-25/2020 Dated 29.02.2020. The normal diet (chow) consists of 5% fats, 53% carbohydrates, and 23% protein. The composition of High Fat Diet was as per Srinivasan et al. (Srinivasan et al. 2005).

**Induction of Type 2 Diabetes**

Streptozotocin (35 mg/kg, *i.p.* in 0.1 M cold citrate buffer, pH-4.4) (Sekar et al. 1990)(Sekar et al. 1990)[8]<sup>8</sup><sup>8</sup>[8]was given to experimental animals after 15 days of diet with high-fat content. Experimental animals reported over 250 mg/dl (16.65 mmol/l) were

considered as diabetic and selected for the study (Srinivasan et al. 2005) (Kulkarni and Garud 2016).

The grouping for the study was as follows.

**Group I:** Normal control group

**Group II:** Diabetic control group

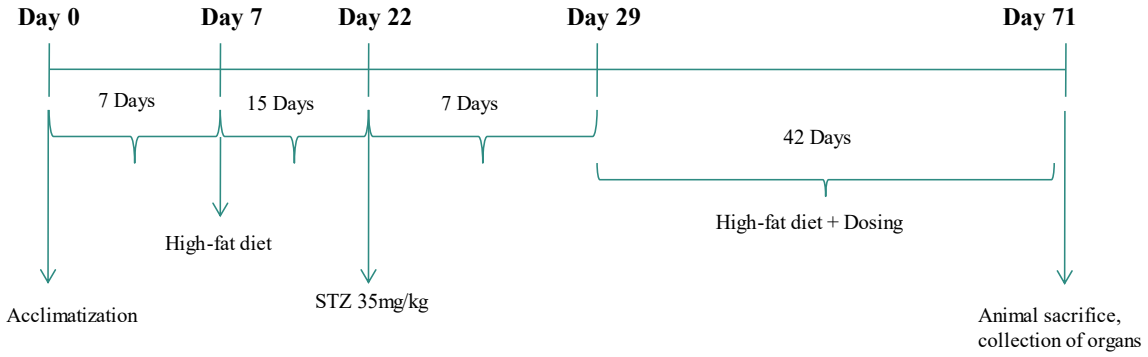
**Group III:** Diabetic rats were given *T. bellirica* fruit extract (500 mg/kg body weight/day/rat) in water for six weeks with oral gavage

**Group IV:** Diabetic rats were given *T. bellirica* fruit extract (1000 mg/kg body weight/day/rat) in water for six weeks with oral gavage

**Group V:** Diabetic rats were administered Glipizide (5 mg/kg body weight/day/rat) in 0.5% Sodium CMC solution for six weeks with oral gavage

**Study Design:**

Animals received a single intraperitoneal injection of STZ at a dose of 35 mg/kg prepared in ice-cold citrate buffer (0.1 M, pH 4.5). Blood glucose was determined 7 days after STZ administration. Animals having blood glucose 250 mg/dL were considered as diabetic and selected for the study.




**Pancreatic Tissue Collection and Processing**

In the end, animals from different treatment groups were humanely sacrificed, and the pancreas was isolated to determine oxidative stress parameters, histopathological studies, and immunohistochemistry.

**Parameters Measured**

**Body weight**

The body weight of the animals from different treatment groups was recorded at the end of the study.

**Determination of Plasma Glucose and Lipids**

Blood samples of animals were collected at the end of the study using EDTA as an anticoagulant. Plasma glucose and lipid markers like HDL, TG, TC, and LDL were determined using commercial diagnostic kits supplied by Transasia Biomedicals Ltd. India by biochemistry analyzer (Erba Chem7, Germany).

**Determination of Liver Enzymes**

A liver enzyme such as AST and ALT were estimated using diagnostic kits supplied by Transasia Biomedicals Ltd. India.

**Measurement of Glycohemoglobin Content**

Glycohemoglobin was determined in freshly collected whole blood samples using a commercially available kit (Transasia Bio-Medicals Ltd., India).

**Measurement of Glucose Tolerance and Insulin Resistance**

***Determination of Oral Glucose Tolerance Test (OGTT)***

OGTT was performed, as reported earlier (Oza and Kulkarni 2018).

***Measurement of Plasma Insulin, Insulin Sensitivity Index, and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR)***

Insulin level was determined in rat plasma samples using enzyme-linked immunosorbent assay (ELISA) kits (Abbkine, USA). Homeostatic Model Assessment- Insulin Resistance (HOMA-IR) and Insulin Sensitivity Index (ISI) were calculated using the following formula: ISI=Ln (1/FINS * FBG)

HOMA-IR = (Glucose X Insulin)/22.5

Where, Concentration of insulin is expressed in mIU/L, Glucose in mmol/l

## Pancreatic Tissue Collection and Processing

### *Determination of Oxidative Stress Parameters*

For oxidative stress parameters, the tissue homogenate was prepared in 10% w/v phosphate buffer (50 mM, pH 7.4). Oxidative Stress Parameters like Superoxide Dismutase (SOD), Catalase (CAT), Reduced Glutathione (GSH), and Malondialdehyde (MDA) were estimated in pancreatic tissue. MDA and GSH levels were measured in the homogenate of pancreatic tissue. CAT was evaluated in the post-nuclear fraction of pancreatic homogenate, whereas SOD was estimated in the post-mitochondrial fraction of homogenate (ELLMAN 1959; Ohkawa, Ohishi, and Yagi 1979; Paoletti, Mocali, and Aldinucci 1990).

### *Histopathology of Pancreatic Tissue*

For histopathology, pancreatic tissue was fixed in 10% buffered formalin. Formalin fixed tissues of pancreas were trimmed longitudinally and routinely processed. Tissue processing was done to dehydrate in ascending grades of alcohol, clearing in xylene and embedded in paraffin wax. Paraffin wax embedded tissue blocks were sections at 3 mm thickness with the Rotary Microtome. All the slides of pancreas were stained with Hematoxylin & Eosin (H & E) stain as per previously reported method for determination of tissue necrosis under a motic microscope (Oza and Kulkarni 2018).

### *Immunohistochemistry*

IHC staining of pancreatic tissue was carried out to determine SIRT1 expression, as reported earlier (Oza and Kulkarni 2018).

## Statistical Analysis

The obtained data are expressed in Mean ± SEM (n=6). GraphPad Prism ver. 5.00 for Windows was used to evaluation of the results. Significant differences between the study groups were assessed by ANOVA (one-way) test followed by Bonferroni's multiple comparison tests to determine the significance level. p-value less than 0.05 were considered significant.

## Results

### Standardization of Ellagic acid and Gallic acid contents in aqueous extract of fruits of *T. bellirica* by HPTLC

The retention factor ($R_f$) of standards of Ellagic acid and Gallic acid was obtained at 0.235 and 0.797, respectively, whereas the peaks of Ellagic acid and Gallic acid in the extract were obtained at a similar retention factor as that of standards confirming the presence of desired tannins in the extract. The % content of Ellagic acid and Gallic acid in the *T. bellirica* aqueous extract was found to be 0.00089531% and 0.017%, respectively. Chromatograms of standards and extract are shown in Figures 1 and 2, and the chromatographic data is presented in Table 1.

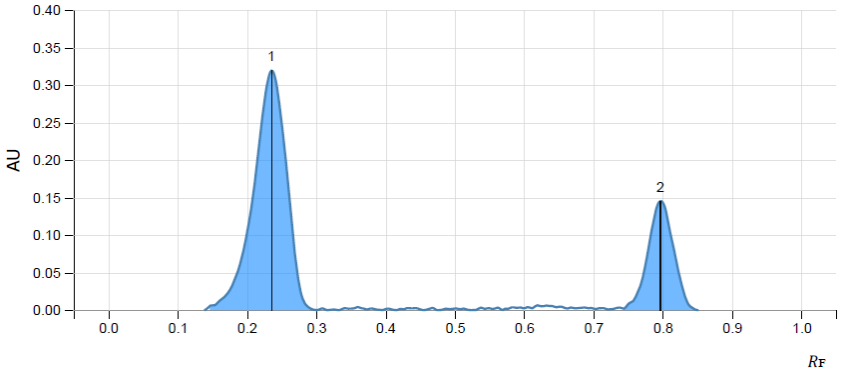



**Figure 1:** Chromatogram of Standard ellagic acid (1) and gallic acid (2).

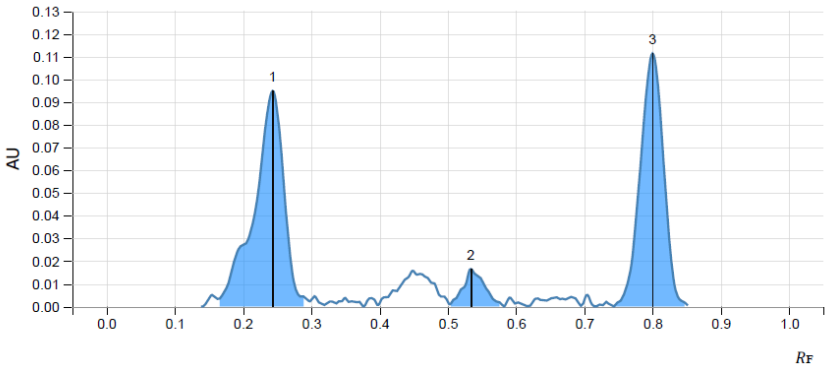



**Figure 2**: Chromatogram of Aqueous extract of fruits of *T. bellirica* showing the presence of Ellagic acid (1) and Gallic acid (3).

**Table 1:** Chromatographic data of Ellagic acid and Gallic acid standards and extract contents.

| Actives | Standard | Tannins in Extract | % Contents of |
|---|---|---|---|

| | Concentration µg/mL | Peak area | Peak area | Concentration µL | **Actives in extracts** |
|---|---|---|---|---|---|
| **Ellagic acid** | 100 | 0.01758 | 0.00477 | 5 | 0.000895 |
| **Gallic acid** | 100 | 0.00626 | 0.00440 | 5 | 0.017 |

## Body Weight

No significant differences were observed in normal control, diabetic control, and treatment group animals (

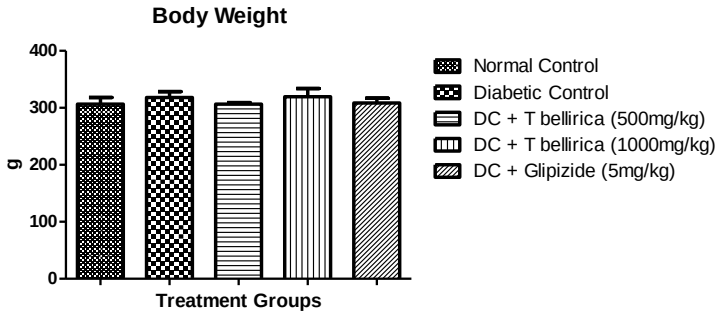



Figure ).

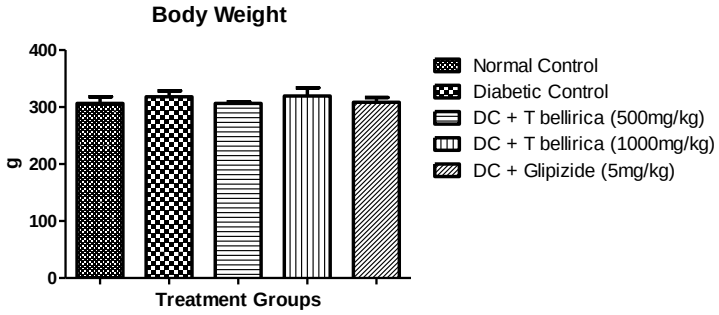



**Figure 3:** Effect of *T. bellirica* aqueous extract on body weight [All values are expressed as Mean ± S.E.M. (n=6)]. (DC: Diabetic Control).

## Determination of Plasma Glucose and Lipid Parameters

### *Plasma Glucose (PG)*

Significantly high Plasma glucose level was reported in the diabetic control group compared with normal animals ($p < 0.001$). *T. bellirica* aqueous extract-treated animals showed a significant

decrease in PG at both doses. At the end of the study, PG levels at both the selected doses are comparable with that of the standard Glipizide (Figure 4).

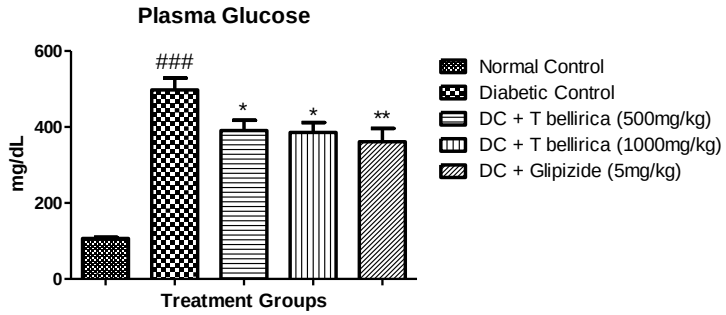
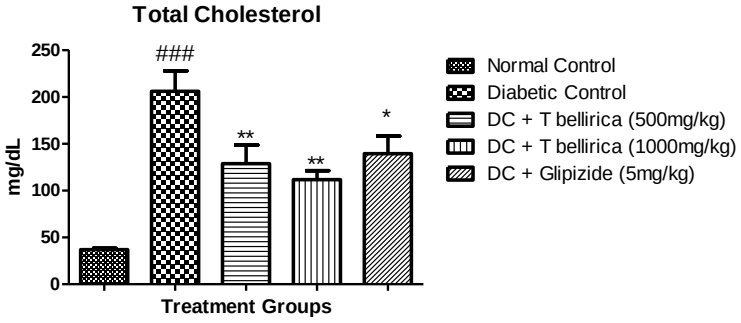



**Figure 4:** Effect of *T. bellirica* extract on plasma glucose. Values are expressed as Mean ± S.E.M. (n=6). $^{*}p < 0.05$, $^{**}p < 0.01$ when treatment groups were compared with diabetic control. $^{###}p < 0.001$ when the diabetic control group compared with the normal control group. (DC: Diabetic Control).

***Lipid Parameters***

Elevated level ($p < 0.001$) of LDL-C, Total Cholesterol, and Triglycerides and reduced level ($p < 0.001$) of Plasma HDL was reported in diabetic experimental animals when compared with the normal control rats. *T. bellirica* treated animals showed a significant decrease in plasma LDL-C, TG, and TC at higher dose levels when compared with the disease control rats. *T. bellirica* treated animals showed improvement in HDL-C level significantly ($p < 0.001$) when compared with the disease control rats (Figure 5).



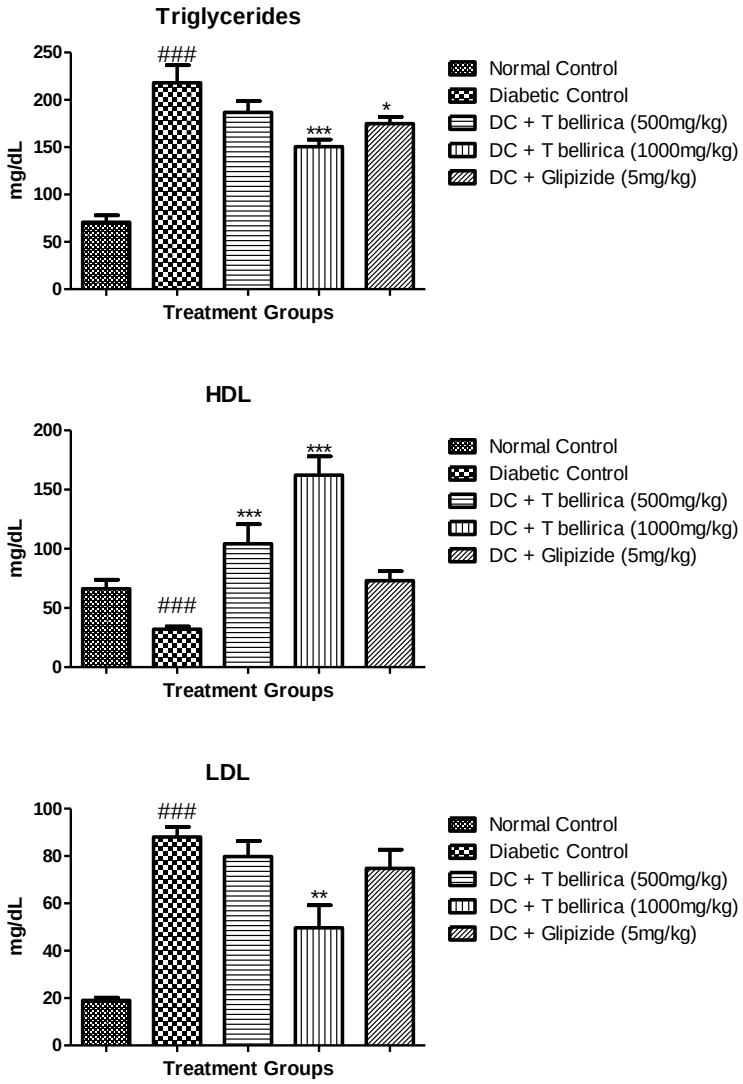



**Figure 5:** Effect of *Terminalia bellirica* treatment on plasma lipids in type 2 diabetic rats. Values are expressed as Mean ± S.E.M. (n=6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when treatment groups were compared with diabetic control. $^{\#\#\#}p < 0.001$ when the diabetic control group compared with the normal control group. (DC: Diabetic Control).

**Effect of *T. bellirica* on Liver Enzymes**

Diabetic control animals showed significant increase in the levels of AST and ALT when compared with normal animals ($p < 0.001$). Extract treatment group, the AST level has been significantly ($p < 0.05$) reduced with the higher dose when compared with the diabetic control group. The aqueous extract also showed a significant reduction in ALT at 500 mg/kg ($p < 0.05$) and 1000 mg/kg ($p < 0.001$) dose levels when compared with diabetic control animals. Glipizide

treatment significantly decreased the level of ALT when compared with disease control animals ($p < 0.01$) (Figure 6).

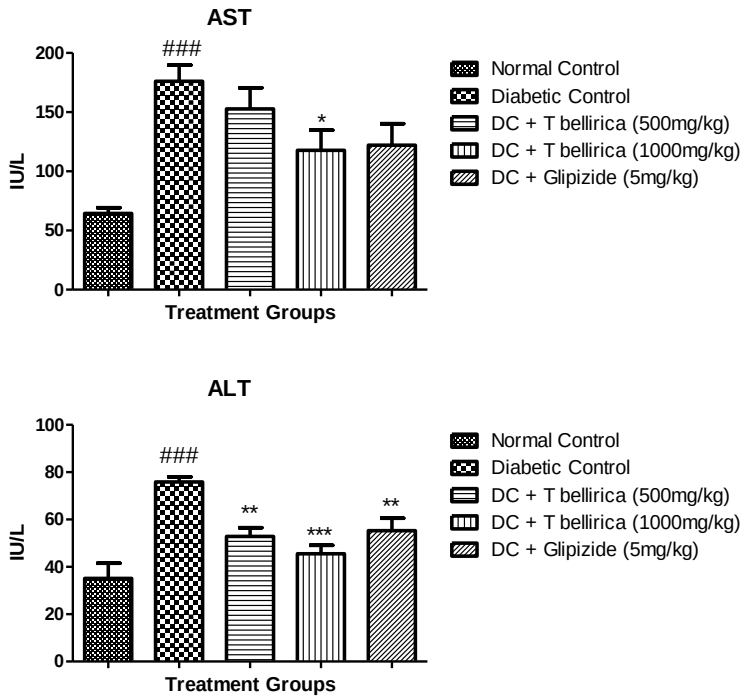



**Figure 6:** Effect of *T. bellirica* on Liver Enzymes. All values are expressed as Mean ± S.E.M. (n=6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when treatment groups were compared with disease control. $^{###}p < 0.001$ when the diabetic control group compared with the normal control. (DC: Diabetic Control).

**Effect of *T. bellirica* on Glycohemoglobin**

Glycohemoglobin concentration was significantly increased in the untreated diabetic group when compared with animals in the normal group. *T. bellirica* reduced Glycohemoglobin levels significantly ($p < 0.001$) at 500 and 1000 mg/kg when compared with the diabetic control group (Figure 7).

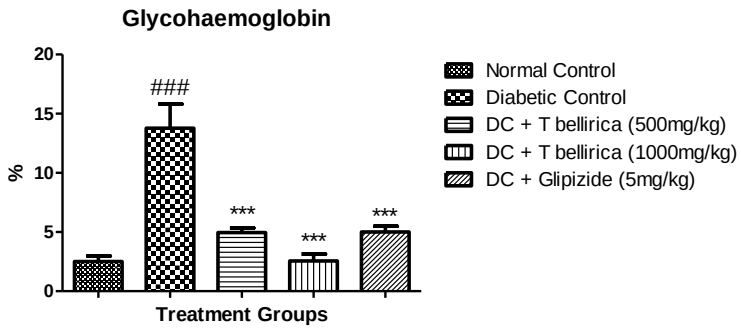



**Figure 7:** Effect of *T. bellirica* treatment on HbA1c (%Hb) in type 2 diabetic rats. All values are expressed as Mean ± S.E.M. (n=6). $^{***}p < 0.001$ when treatment groups were compared with disease control rats. $^{###}p < 0.001$ when the disease control group compared with the normal control. (DC: Diabetic Control).

**Determination of Glucose Tolerance and Insulin Resistance Measurement**

***Effect of T. bellirica on Oral Glucose Tolerance Test (OGTT)***

Oral glucose tolerance was performed at the end of the study. The plasma glucose level was found to remain significantly high in diabetic animals ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$), while diabetic animals treated with aqueous extract of *T. bellirica* significantly improved glucose tolerance when compared with untreated diabetic animals (Figure 8).

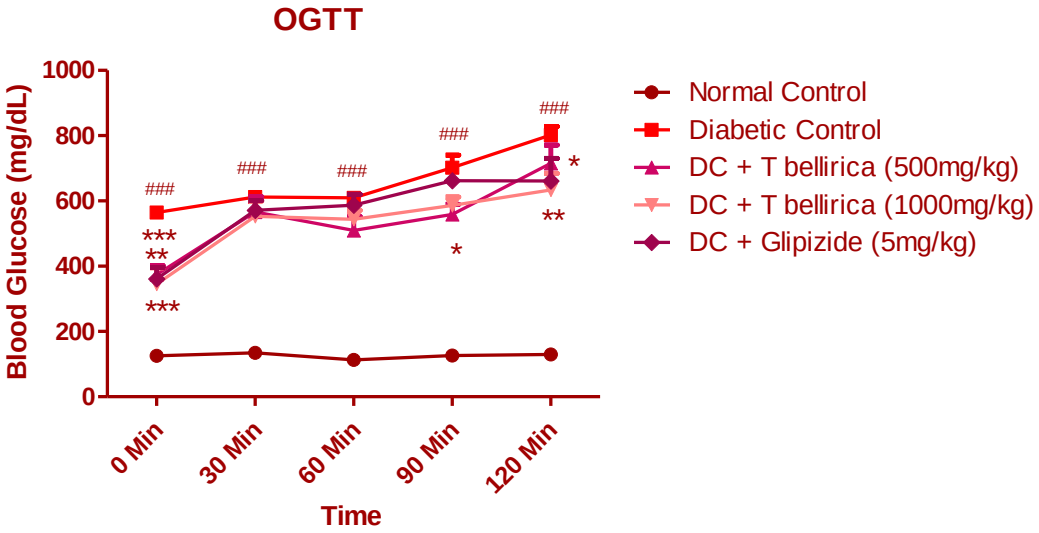
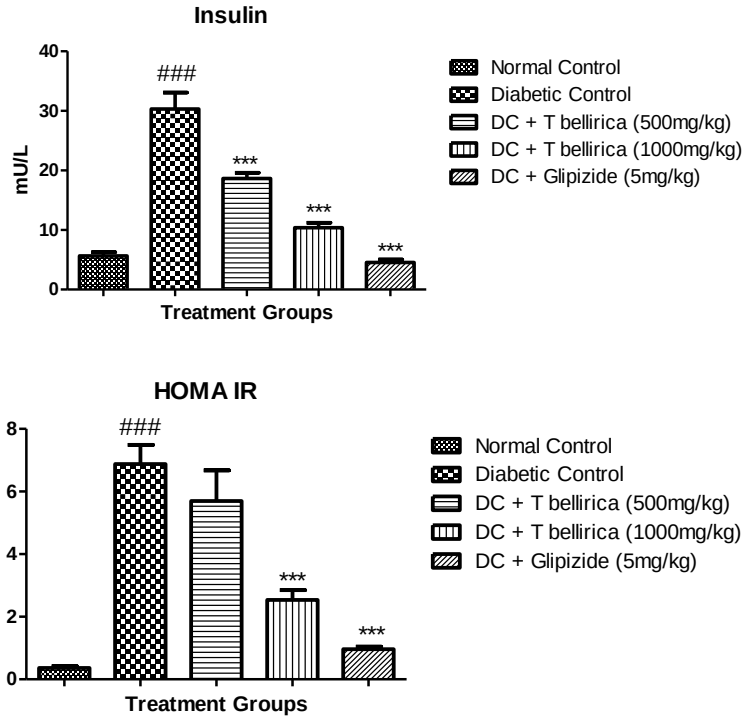


**Figure 8**: Effect of *Terminalia bellirica* extract on OGTT. All values are expressed as Mean ± S.E.M. (n=6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when treatment groups were compared with disease control. $^{###}p < 0.001$ when the disease control group compared with normal control. (DC: Diabetic Control).

## Measurement of Plasma Insulin (PI), Insulin Sensitivity Index (ISI), and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR)

Untreated diabetic animals showed a significant increase in the plasma insulin level at the end of the study of diabetic induction when compared with normal animals. At the same time, diabetic animals treated with *T. bellirica* aqueous extract showed significant reduction ($p<0.001$) in plasma insulin levels. The treatment also showed a significant reduction in insulin resistance and improved insulin sensitivity at all selected doses (Figure 9).



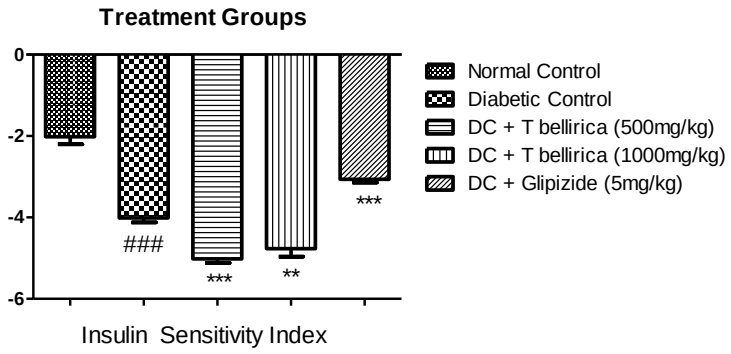
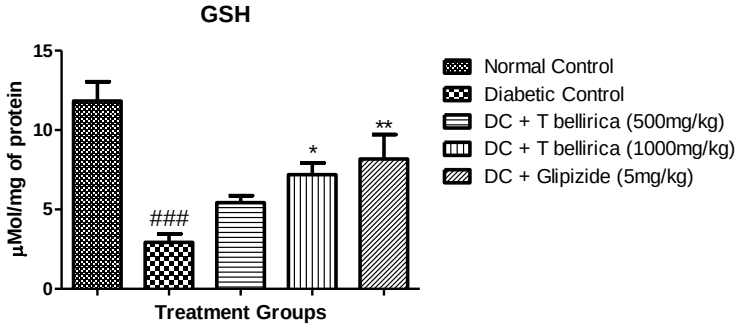



**Figure 9:** Effect of *Terminalia bellirica* treatment on Plasma Insulin, HOMA-IR, and Insulin Sensitivity Index (ISI) in type 2 diabetic rats. Values are expressed as Mean ± S.E.M. (n=6). $^{***}p < 0.001$ when treatment groups were compared with diabetic control. $^{###}p < 0.001$ when the diseased control group compared with the normal control group. (DC: Diabetic Control).

## Pancreatic Tissue Collection and Processing

### *Effect of T. bellirica on Oxidative Stress Parameters*

Diabetic group reading showed a significant decrease in antioxidant enzymes viz., GSH, SOD, CAT, and a significantly increased MDA level in pancreatic tissue when compared to normal animals ($p < 0.001$). A high dose of *T. bellirica* significantly prevented the loss of GSH ($p < 0.05$) when compared with the diabetic group. *T. bellirica* treatment at selected dose levels does not significantly prevented the loss of SOD. High dose *T. bellirica* treatment dose significantly avoids the loss of CAT ($p < 0.05$) when compared with the diabetic group. *T. bellirica* treatment at a high dose and standard Glipizide treatment significantly prevented ($p < 0.01$ and $p < 0.05$) the formation of MDA in pancreatic tissue homogenate when compared with the diabetic control group (Figure 10).



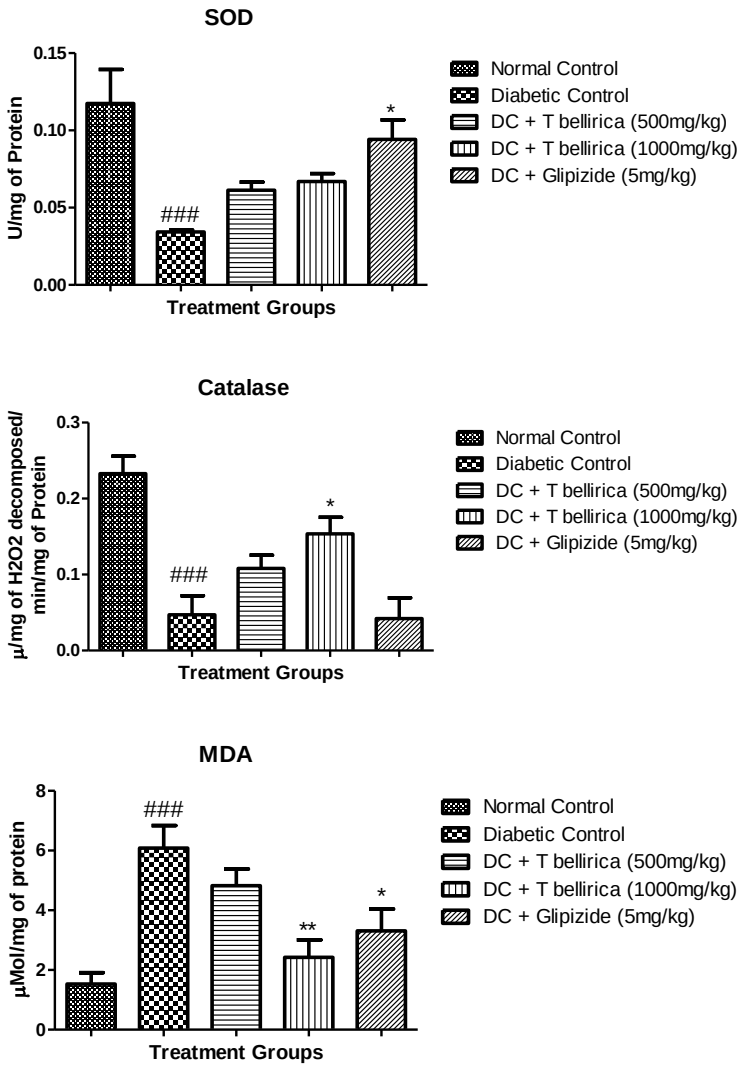



**Figure 3:** Effect of *Terminalia bellirica* aqueous extract on Oxidative Stress Parameters. All values are expressed as Mean ± S.E.M. (n=6). $^{*}p < 0.05$, $^{**}p < 0.01$ when treatment groups were compared with disease control. $^{###}p < 0.001$ when the disease control group compared with the normal control group. (DC: Diabetic Control).

### *Effect of T. bellirica on histopathology of pancreatic tissue*

Pancreatic tissue of the diabetic animals showed hyperplastic acini in the exocrine region while degeneration and atrophy of Langerhans islet as compared to normal animals' pancreatic tissue. Adipose tissue deposition at the acini lobe, hyperplastic acini, and necrosis of peri-parenchymal adipose tissue was observed in diabetic animals treated with 1000 mg/kg of aqueous extract. The severity of the lesions is shown in Figure 11.

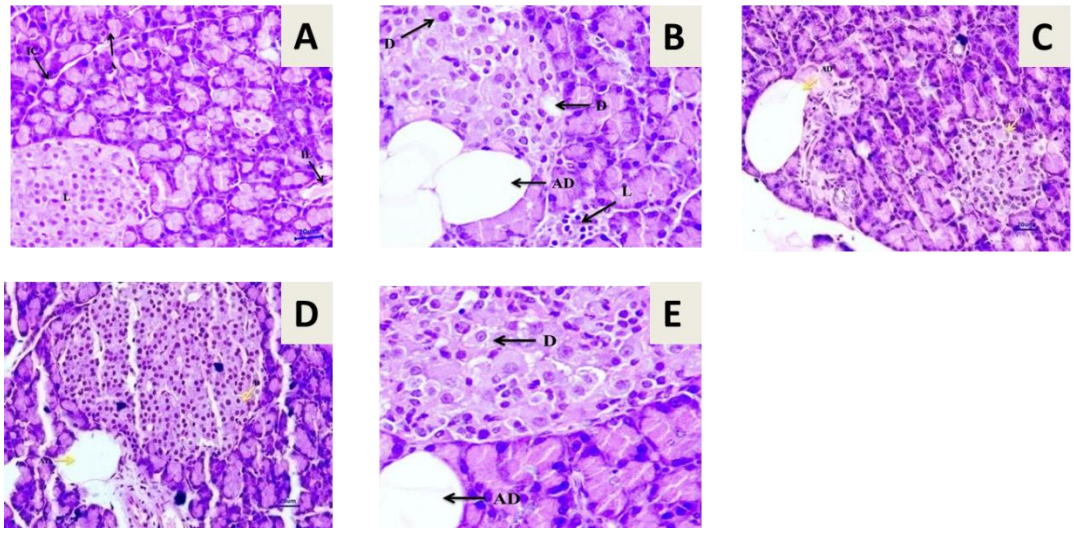



**Figure 41:** Effect of *Terminalia bellirica* on pancreatic tissue histology (H & E Stain, 400X) [A: Normal Control, B: Diabetic Control, C: DC + *T. bellirica* (500mg/kg), D: DC + *T. bellirica* (1000mg/kg), E: DC + Glipizide (5mg/kg)]

***Effect of T. bellirica extract on SIRT1 expression in pancreatic tissue***

Untreated diabetic animals showed a reduction in the optical density of immune-stained pancreatic tissue compared to normal animals showing a reduction in SIRT1 expression in pancreatic tissue. Treatment with 500 and 1000 mg/kg of the extract showed a significant increase in SIRT1 expression in pancreatic tissue (Figure 12).

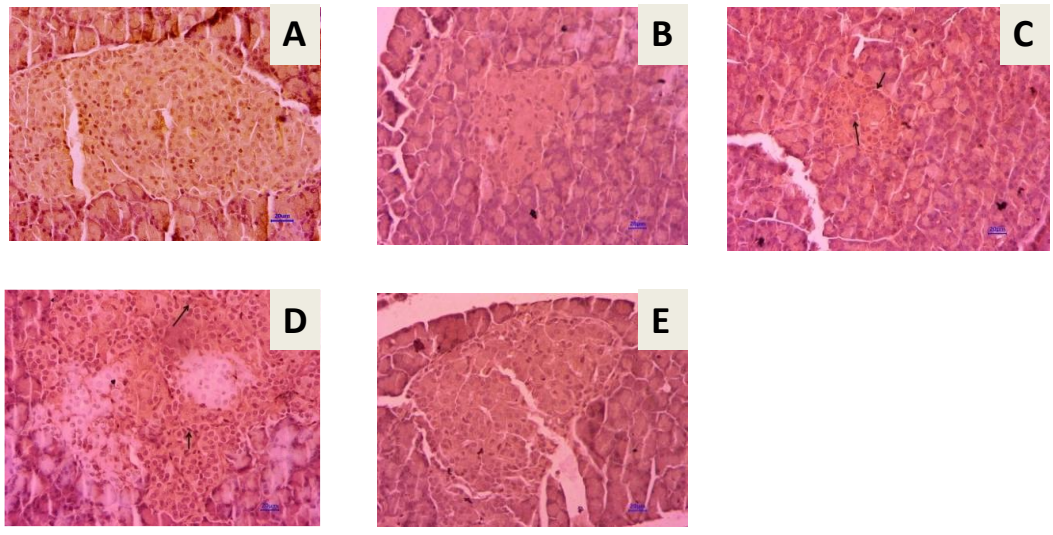


**Figure 52:** Immunohistochemical staining of the pancreas (400 X). Arrow showed expression of SIRT1 in the Islet of Langerhans. [A: Normal Control, B: Diabetic Control, C: DC + *T. bellirica* (500mg/kg), D: DC + *T. bellirica* (1000mg/kg), E: DC + Glipizide (5mg/kg)]

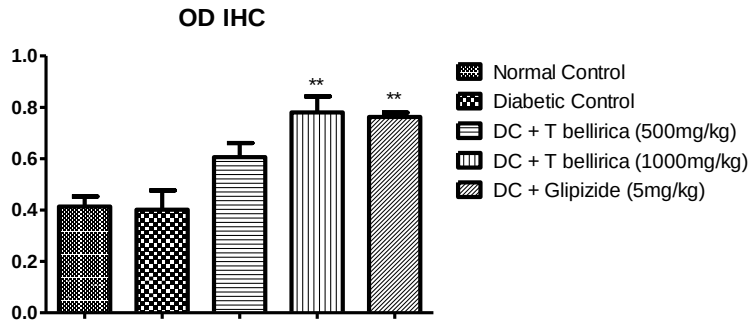



**Figure 6:** Change in Optical density for expression. All values are expressed as Mean ± S.E.M. (n=3). $^{**}p < 0.05$ when treatment groups were compared with disease control. (DC: Diabetic Control).

**Conclusion:**

The result of present study indicates that *Terminalia bellirica* extract has significant antidiabetic effect.

**II Aim:** To study the effect of *Terminalia chebula* extract on experimental model of Type 2 Diabetes Mellitus.

## Materials and methods

### Plant Materials

The dried fruits of *T. chebula* were procured from the D.G. Ayurvedic Sangrah, Mumbai, India in November 2018. The plant material was authenticated by Dr. (Mrs.) A. S. Upadhye, Scientist, Herbal Drug Administration at Agharkar Research Institute, Pune, Maharashtra, India. A voucher specimen (Auth. 18-205) has been deposited in the Department for future use.

### Chemicals

Yeast extract and DL-methionine were obtained from Molychem, India. Cholesterol was obtained from Loba Chemie Pvt. Ltd., India. Casein was procured from Clarion casein limited, India. The lard was obtained from the local vendor. Streptozotocin was ordered from Sigma Aldrich (USA), and sodium chloride was purchased from SD Fine Chemicals, India. From Transasia Biomedicals Ltd, India, diagnostic kits were obtained. Glipizide was purchased from the local market.

### Preparation of Aqueous Extract

Kernels of dried fruits of *T. chebula* were removed, powdered, and used for the preparation of aqueous extract by using the double maceration technique. Powdered fruits (500 g) were macerated with 5000 mL of distilled water for seven days. At the end of the seventh day, the extract was filtered through muslin cloth, and the filtrate was stored in the refrigerator till the subsequent processing. The marc was again kept for maceration with fresh distilled water for seven days. The extract was filtered through muslin cloth after seven days. The filtrate obtained in this step was mixed with the filtrate obtained in the first step. The extract was concentrated in a water bath (Make: Labline, Mumbai, India) and was stored in a refrigerator (Make: Toshiba, Model: UD-IND-SX, India) until further use (Kulkarni et al. 2013). The percentage yield of semisolid extract was found to be around 34%w/w.

### Quantification of ellagic acid and gallic acid in the aqueous extract by HPTLC method

Quantification of ellagic acid and gallic acid from the aqueous extract of *T. chebula* was done by the High-Performance Thin Layer Chromatography (HPTLC) method. HPTLC analysis was

carried out by using CAMAG HPTLC (Switzerland) equipped with visionCATS HPTLC Software, CAMAG Linomat V applicator, CAMAG Hamilton Micro syringe (100 µL), CAMAG TLC Scanner 4 (4.6 mm×25 cm, 5 µm), CAMAG UV Cabinet (254 & 366 nm), CAMAG Twin Trough Chambers (20 x 10 cm). The separation was achieved using a solvent system of *Methanol: Water: Formic acid (4:6:0.5) v/v/v*, and the stationary phase was pre-coated alumina backed, HPTLC Plate Silica gel 60 RP-18 (Merck). The scanning was done at 254 nm. Ellagic acid and gallic acid reference material were used as standards for quantifying tannins in the extract.

**Preparation of Standard Solutions**

The standard solutions of ellagic acid and gallic acid of the concentration of 100 µg/mL were prepared in methanol and applied to the HPTLC system.

**Experimental animals**

Male Sprague Dawley rats (160–170 g) were purchased from the National Institute of Biosciences, Pune, India. These animals were housed in the animal facility at a temperature and relative humidity of 22 ± 2°C and 75 ± 5%, respectively. The experimental animals were fed a basal regular pellet diet (Nutrimix Laboratory Animal Feed, MS, India) throughout the experiment. The high-fat diet consists of 25% protein, 58% fat, and 17% carbohydrate as a percentage of total kcal (Srinivasan et al. 2005). The high-fat diet composition per kilogram includes Normal pellet diet 365 g, Pork lard 310 g, Casein 250 g, Cholesterol 10 g, Vitamin D mineral mix 60 g, DL-Methionine 03 g, Yeast powder 01 g, Sodium Chloride 01 g. The Institutional Animal Ethics Committee approved the animal study protocol of Shri Vile Parle Kelavani Mandal on 29$^{th}$ February 2020 (Protocol number CPCSEA/IAEC/P-25/2020). Considering the mortality during the study, number of animals per group were kept 10 so that minimum six observations can be obtained for evaluation.

**Induction of Diabetes**

Type 2 diabetes was induced using a low dose of Streptozotocin (STZ) (35 mg/kg, i.p., using 0.1 M cold citrate buffer at pH - 4.4, volume based upon body weight) (Rana, Singh, and Mehan 2018) (Kalaivani and Sankaranarayanan 2019) after two weeks of dietary modification using a high-fat

diet (Srinivasan et al. 2005). The experimental animals having blood glucose levels above 250 mg/dL were selected for the research study (Kulkarni and Garud 2016).

**Group I:** Normal control (received purified water)

**Group II:** Diabetic control rats (received purified water)

**Group III:** Diabetic rats were given *T. chebula* fruits aqueous extract (500 mg/kg body weight/day/rat) in distilled water.

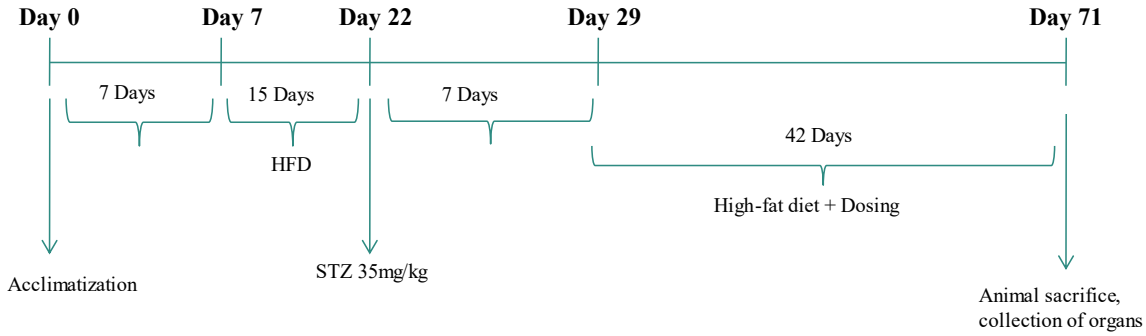
**Group IV:** Diabetic rats were given *T. chebula* fruits aqueous extract (1000 mg/kg body weight/day/rat) in distilled water.

**Group V:** Diabetic rats were administered with Glipizide (standard drug) (5 mg/kg body weight/day/rat) in 0.5% Sodium Carboxy Methyl Cellulose (Na CMC)

The treatment was given for six weeks by oral route with the help of oral gavage during the day time in between 10 AM to 11 AM. All animals in animal house facility are supplied with purified water.

**Study Design:**

Animals received a single intraperitoneal injection of STZ at a dose of 35 mg/kg prepared in ice-cold citrate buffer (0.1 M, pH 4.5). Blood glucose was determined 7 days after STZ administration. Animals having blood glucose 250 mg/dL were considered as diabetic and selected for study.




**Pancreatic Tissue Collection and Processing**

In the end, animals from different treatment groups were humanely sacrificed, and the pancreas was isolated to determine oxidative stress parameters, histopathological studies, and immunohistochemistry.

**Evaluation of Parameters**

**Body weight**

The body weight of the animals from different groups was recorded at the end of the study.

**Estimation of Plasma Glucose and Lipids**

Blood samples of animals were collected, and plasma was separated. Plasma glucose level and lipid markers like total cholesterol, triglyceride, high-density lipoprotein, and low-density lipoprotein were performed by kits procured from Transasia Bio-Medicals Ltd., India, and evaluation was done using a Biochemical Analyzer, namely Erba Chem-7, Germany.

**Estimation of Liver Enzymes**

Alanine Transaminase and Aspartate Aminotransferase were measured per the protocol mentioned in the leaflets of the kits supplied by Transavia Bio-Medicals Ltd., India.

**Estimation of Glycohemoglobin Content**

Using the resin (ion exchange) method, as per the procedure mentioned in the kit supplied by Transasia Bio-Medicals Ltd., India, Glycohemoglobin content was determined.

**Estimation of Glucose Tolerance and Insulin Resistance**

**Estimation of Oral Glucose Tolerance Test (OGTT)**

An OGTT was performed as per the previously described method (Kulkarni and Garud 2016).

**Measurement of Plasma Insulin (PI), Insulin Sensitivity Index (ISI), and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR)**

Insulin level was estimated in plasma samples using rat insulin enzyme-linked immunosorbent assay (ELISA) kits supplied by Abbkine, USA.

Homeostatic Model Assessment- Insulin Resistance (HOMA-IR) and Insulin Sensitivity Index (ISI) were calculated using the following formula:

$$ISI = \ln (1 / FINS * FBG)$$

$$HOMA\text{-}IR = (Glucose\ X\ Insulin) / 22.5$$

Where,

The concentration of insulin is expressed in mIU/L, Glucose in mmol/l

**Pancreatic Tissue Collection and Processing**

**Determination of Oxidative Stress Parameters**

Tissue homogenate (10% w/v) was prepared in phosphate buffer 50 mM (pH 7.4). Oxidative stress parameters like Malondialdehyde, Superoxide Dismutase, Catalase, and Reduced Glutathione were studied in pancreatic tissue homogenate. MDA and GSH levels were also estimated in pancreatic tissue homogenate. CAT was evaluated in the post-nuclear homogenate fraction, whereas SOD was assessed in the post-mitochondrial fraction of homogenate (Ellman, 1959; Ohkawa, Ohishi, and Yagi, 1979; Paoletti, Mocali and Aldinucci, 1990) (Garud and Kulkarni 2017).

**Histopathology of Pancreatic Tissue**

The study was processed as reported earlier (Kulkarni and Garud 2016) (Oza and Kulkarni 2018). The stained slides were studied under a motic microscope for histopathological changes.

**Immunohistochemistry**

As reported earlier, the pancreatic tissue immunohistochemical staining was carried out to express SIRT1 (Oza and Kulkarni 2018).

## Statistical analysis

The estimation was done using software, namely, GraphPad Prism for Windows ver. 5.00. The data are expressed in Mean ± SEM. Significant differences between the experimental animal groups were assessed by one-way analysis of variance test (ANOVA) followed by Bonferroni's multiple comparison tests to determine the significance level. *p*-value less than 0.05 was considered significant.

**Results**

**Identification of ellagic acid and gallic acid in aqueous extract of fruits of *T. chebula* by HPTLC Method**

Using the mobile phase mentioned in the materials and method section, we have reproducible and sharp ellagic and gallic acid peaks with symmetry as per the limit. Hence, it was considered an

optimized condition for standardizing ellagic and gallic acid in the extract. The retention factor for standard ellagic acid and gallic acid was obtained at 0.234 and 0.795, respectively, whereas the peaks of ellagic acid and gallic acid in the extract were obtained at a similar retention factor as that of standards, confirming the presence of desired tannins in the extract. The percent content of ellagic acid and gallic acid in the *T. chebula* aqueous extract was found to be 0.0018% and 0.0077%, respectively. Chromatograms of standards and extract are shown in Figure 1 and Figure 2, and the chromatographic data is presented in Table 1.

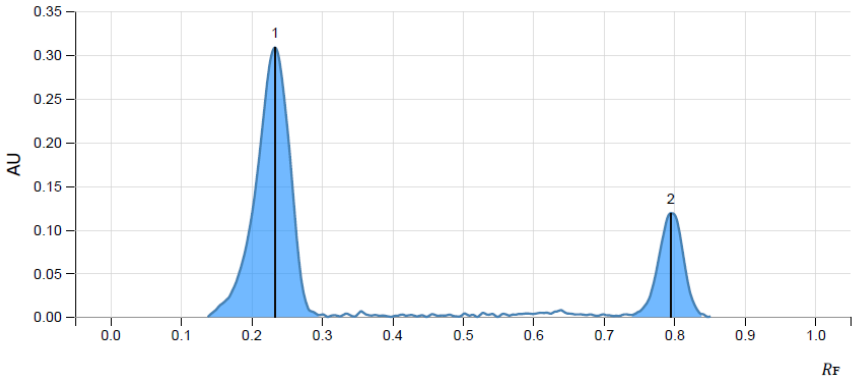



**Figure 7**: Chromatogram of Standard ellagic acid (1) and gallic acid (2).

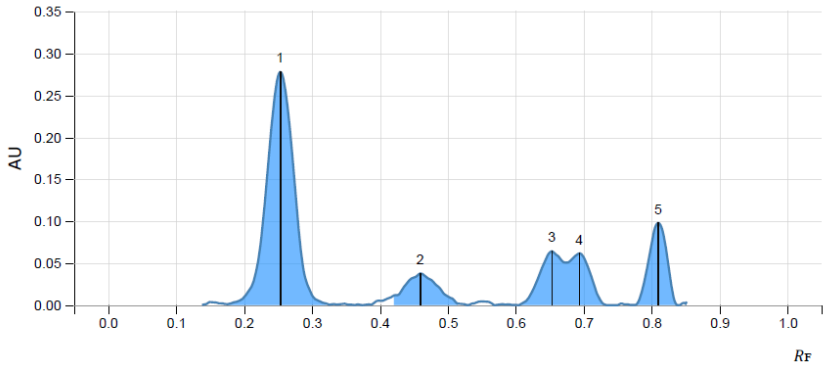



**Figure 8**: Chromatogram of Aqueous extract of fruits of *T. chebula* showing the presence of ellagic acid (1) and gallic acid (5).

**Table 1.** Chromatographic data of ellagic acid and gallic acid standards and extract contents.

| **Actives** | **Standards** | | **Tannins in Extract** | | **% Contents of actives in extracts** |
|---|---|---|---|---|---|
| | Concentration µg/mL | Peak area | Peak area | Concentration µL | |
| Ellagic acid | 100 | 0.01724 | 0.01280 | 8 | 0.0018 |

| | | | | | |
|---|---|---|---|---|---|
| Gallic acid | 100 | 0.00498 | 0.00298 | 8 | 0.0077 |

**Body weight**

The effect of *T. chebula* on the body weight of animals of different treatment groups. When we compared the diseased control group, a considerable difference was not observed when compared with normal, diabetic control, and treatment groups (Figure 3).

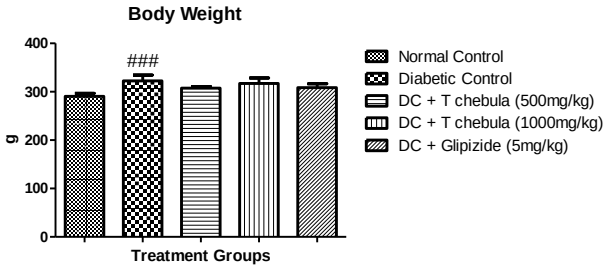



**Figure 9:** Effect of *T. chebula* extract on body weight. Values are expressed as Mean ± S.E.M. (n = 6). $^{###}p < 0.001$ when the diabetic control group compared to the normal control group. (DC: Diabetic Control; *T chebula*: *Terminalia chebula*).

## Effect of *Terminalia chebula* extract on Plasma Glucose and Lipid Parameters in diabetic rats

**Plasma Glucose**

Diabetic rats showed a significantly ($p < 0.001$) high plasma glucose level compared to the normal group. The *T. chebula* treatment group showed a significant ($p < 0.001$) reduction in plasma glucose at 500 mg/kg and 1000 mg/kg doses compared with the diabetic control group. The results at both dose levels of *T. chebula* (i.e., 500 and 1000 mg/kg) are similar and comparable with the Glipizide (Figure 4).

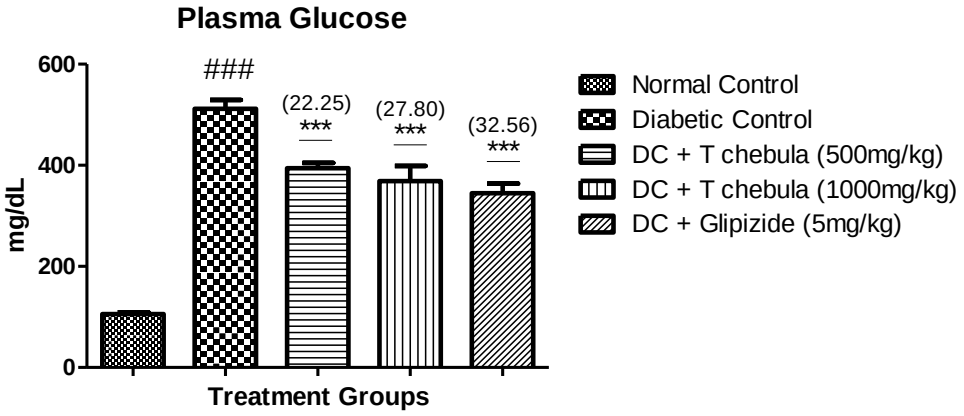



**Figure 10:** Effect of *T. chebula* extract on Plasma Glucose. Values are expressed as Mean ± S.E.M. (n=6). $^{***}p < 0.001$ when treatment groups were compared with diabetic control. $^{###}p < 0.001$ when the diabetic control group compared to the normal control group. (DC: Diabetic Control; *T chebula*: *Terminalia chebula*). Values in parenthesis indicate percentage reduction.

**Lipid Parameters**

The effect of *T. chebula* on plasma levels of triglyceride, total cholesterol, LDL, and HDL has shown in Figure 5. The diabetic control group showed a significant ($p < 0.001$) increase in the plasma TC, TG, and LDL levels compared with the normal control group. The plasma HDL level was also significantly lower in a diabetic control animal ($p < 0.001$) than in normal animals. Treatment with aqueous extract at 1000 mg/kg significantly reduced triglyceride levels ($p < 0.001$) in diabetic animals when compared with the diabetic control group. Total cholesterol level was also markedly reduced in diabetic animals ($p < 0.001$) after treatment with *T. chebula* aqueous extract at 500 and 1000 doses. *T. chebula* aqueous extract at both doses showed a significant increase in HDL level ($p < 0.001$) in diabetic rats. Treatment with *T. chebula* aqueous extract showed a significant reduction in LDL levels at both doses ($p < 0.001$).

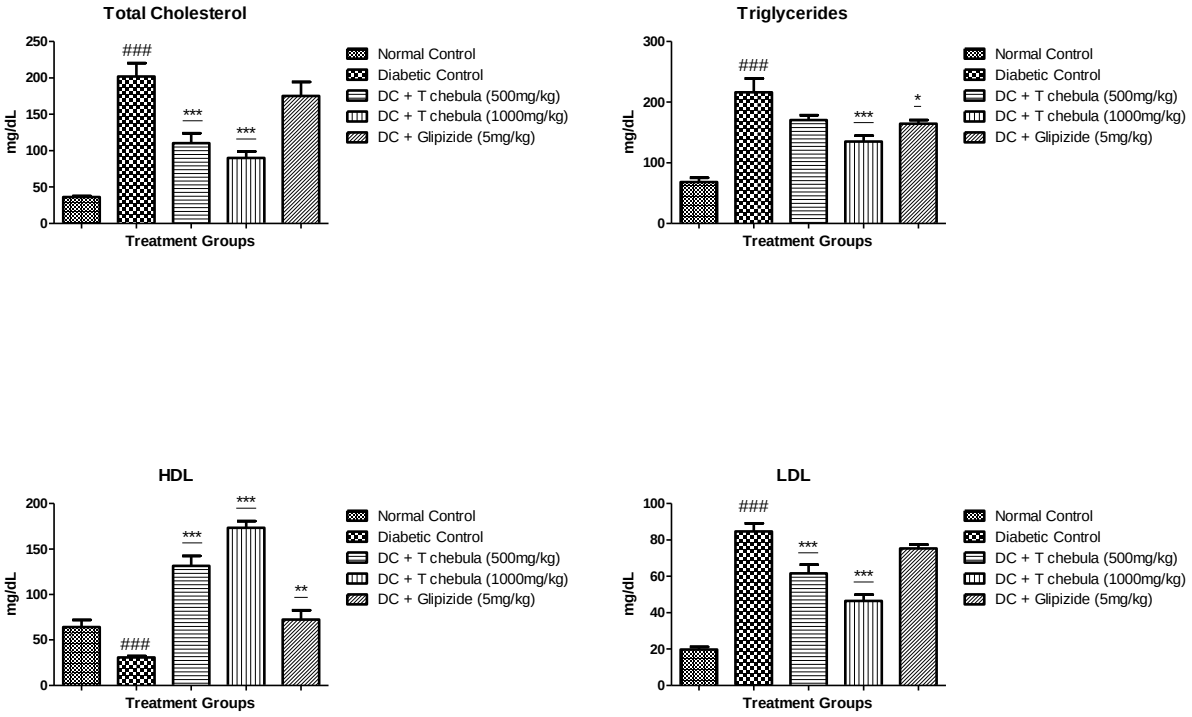
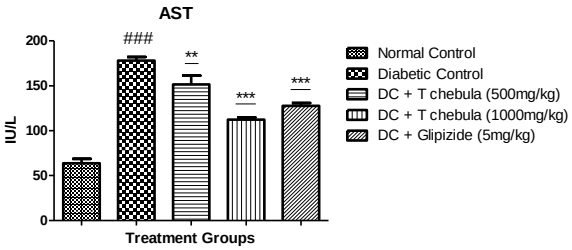



**Figure 11:** Effect of *T. chebula* treatment on plasma lipids in type 2 diabetic rats. Values are expressed as Mean ± S.E.M. (n=6)., $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when treatment groups were compared with diabetic control. $^{###}p < 0.001$ when the diabetic control group compared to the normal control group. (DC: Diabetic Control; *T chebula*: *Terminalia chebula*).

**Effect of *T. chebula* extract on Liver Enzymes**

Diabetic animals showed a significant ($p < 0.001$) increase in levels of AST and ALT in plasma compared to normal rats. Treatment with *T. chebula* aqueous extract at 500 mg/kg ($p < 0.01$) and 1000 mg/kg ($p < 0.001$) dose showed a significant reduction of AST when compared with diabetic control rats. Treatment with *T. chebula* aqueous extract significantly reduces ALT at 500 mg/kg ($p < 0.05$) and 1000 mg/kg ($p < 0.001$) dose when compared with diabetic control rats. Standard Glipizide treatment significantly ($p < 0.05$) reduced the level of ALT in comparison with disease control rats (Figure 6).


AST
200
150
100
50
0
IU/L
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**
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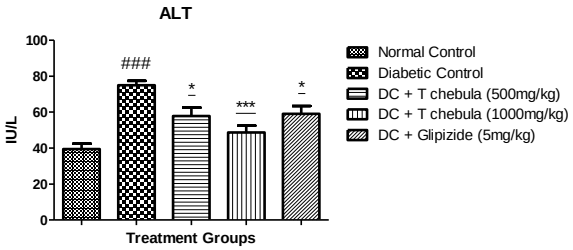
Treatment Groups
Normal Control
Diabetic Control
DC + T chebula (500mg/kg)
DC + T chebula (1000mg/kg)
DC + Glipizide (5mg/kg)


ALT
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80
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Treatment Groups
Normal Control
Diabetic Control
DC + T chebula (500mg/kg)
DC + T chebula (1000mg/kg)
DC + Glipizide (5mg/kg)

**Figure 12:** Effect of *T. chebula* on Liver Enzymes. All values are expressed as Mean ± S.E.M. (n=6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when treatment groups were compared with disease control. $^{\#\#\#}p < 0.001$ when the diabetic control group compared with the normal control group. (DC: Diabetic Control; *T chebula*: *Terminalia chebula*).

**Effect of *T. chebula* on Glycohemoglobin**

Diabetic animals showed a significant increase ($p < 0.001$) in glycohemoglobin levels in the blood when compared with the normal group. While treatment with *T. chebula* aqueous extract at two selected doses showed a significant reduction ($p < 0.001$) in glycohemoglobin level in blood when compared with the diabetic control group (Figure 7).

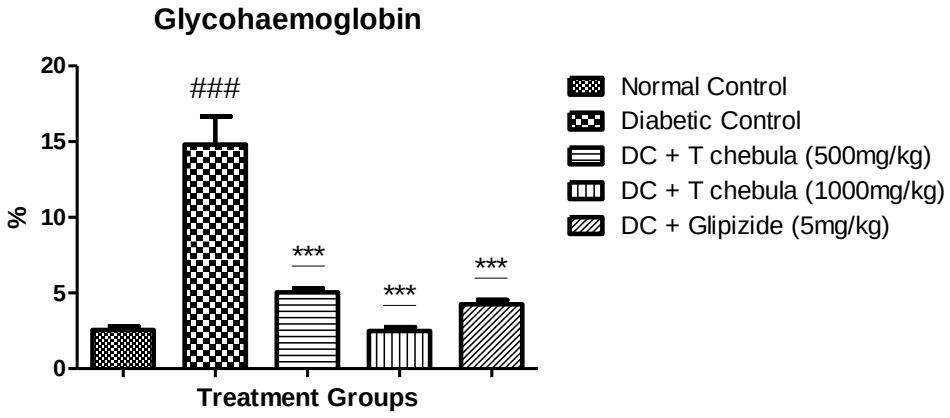



**Figure 13:** Effect of *T. chebula* treatment on HbA1c (%Hb) in type 2 diabetic rats. Values are expressed as Mean ± S.E.M., $^{***}p < 0.001$ when the treatment group is compared with the diabetic control group. $^{\#\#\#}p < 0.001$ when the diabetic control group compared to the normal control. (DC: Diabetic Control; *T chebula*: *Terminalia chebula*).

**Glucose tolerance and insulin resistance measurement**

**Effect of *T. chebula* on Oral Glucose Tolerance Test (OGTT)**

Figure 8 illustrates the result of the oral glucose tolerance test performed at the end of the study in 8-hour fasted rats. The results showed that the plasma glucose level at 0, 30, 60, 90, and 120 min remained significantly higher in the diabetic control group compared with the normal control group ($p < 0.001$) and other treatment groups ($p < 0.05$ and 0.01). The OGTT curve declined gradually at all selected *T. chebula* aqueous extract treatment doses.

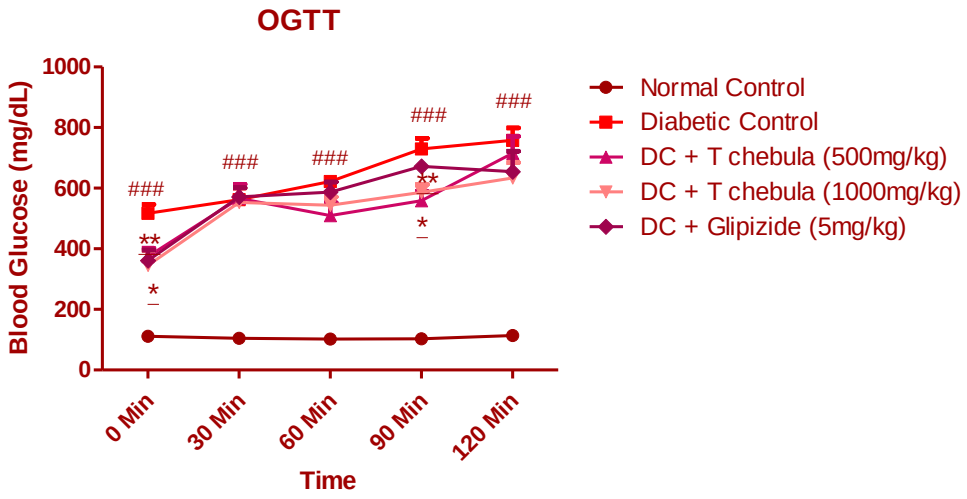



**Figure 14:** Effect of *T. chebula* aqueous extract on OGTT. Values are expressed as Mean ± SEM (n=6). $^{*}p < 0.05$, $^{**}p < 0.01$ when treatment groups were compared with disease control. $^{###}p < 0.001$ when the diabetic control group compared with the normal control groups. (DC: Diabetic Control; *T chebula*: *Terminalia chebula*).

**Measurement of Plasma Insulin (PI), Insulin Sensitivity Index (ISI), and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR)**

Plasma insulin level was increased significantly in diabetic rats, while treatment with *T. chebula* aqueous extract reduced plasma insulin level significantly at 500 and 1000 mg/kg ($p < 0.001$). In addition, *T. chebula* aqueous extract treatment also improved insulin sensitivity by significantly ($p < 0.001$) decreasing HOMR-IR and increasing the insulin sensitivity index (ISI) of the treated rats (Figure 9).

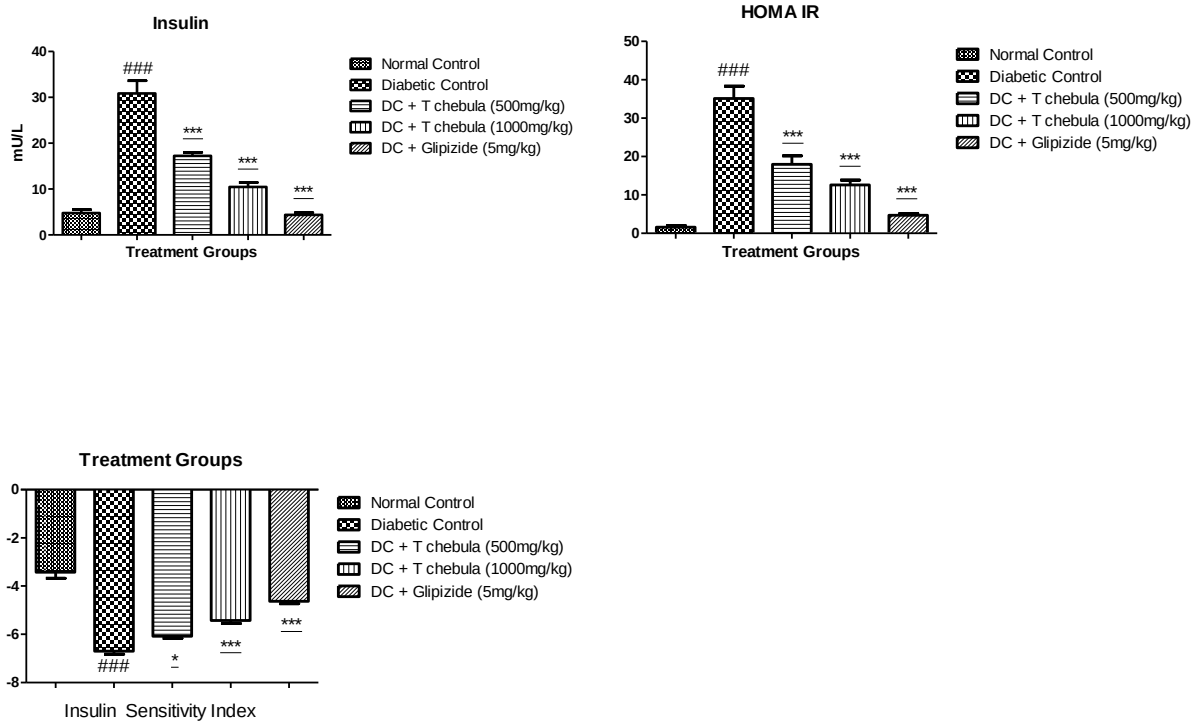



**Figure 15:** The *T. chebula* aqueous extract treatment Effect on A. Plasma Insulin B. HOMA-IR C. Insulin Sensitivity Index (ISI) in type 2 diabetic rats. Values are expressed as Mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when treatment groups were compared with diabetic control. $^{\#\#\#}p < 0.001$ when the diabetic control group compared to the normal control group. (DC: Diabetic Control; *T chebula*: *Terminalia chebula*).

**Effect of *T. chebula* treatment on oxidative stress parameters**

Diabetic rats showed a significant decrease in the levels of antioxidant enzymes like SOD, GSH, and CAT and a substantial increase in the level of MDA in pancreatic tissue homogenate compared to normal rats ($p < 0.001$). Treatments with a high dose of *T. chebula* aqueous extract significantly prevent the loss of GSH ($p < 0.05$) compared with diabetic control rats. *T. chebula* treatment at 1000 mg/kg significantly ($p < 0.01$) prevents the loss of CAT. Low and high dose of *T. chebula* aqueous extract treatment does not significantly prevent the loss of SOD when compared with diabetic control rats. *T. chebula* treatment at 1000 mg/kg dose and standard Glipizide treatment significantly ($p < 0.01$) prevent the formation of MDA in pancreatic tissue homogenate when compared with diabetic control rats (Figure 10).

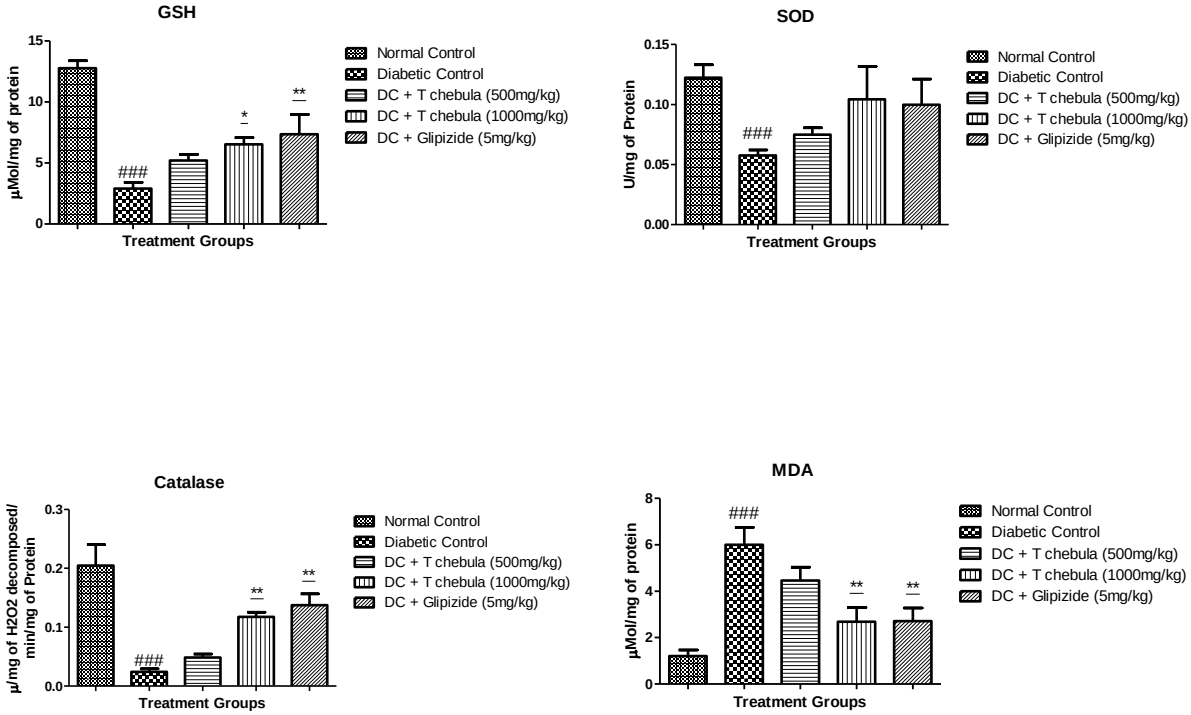



**Figure 16:** Effect of *T. chebula* aqueous extract on oxidative stress parameters. All values are expressed as Mean ± S.E.M. (n=6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when treatment groups were compared with disease control. $^{\#\#\#}p < 0.001$ when the diabetic control group compared with the normal control group. (DC: Diabetic Control; *T chebula*: *Terminalia chebula*).

**Effect of *T. chebula* on histopathology of pancreatic tissue**

Microscopic examination of the pancreatic tissues of rats from the normal control group did not reveal any lesion of pathological significance (Figure 11). Rats from the diabetic control group and treated groups showed various lesions in pancreatic tissue.

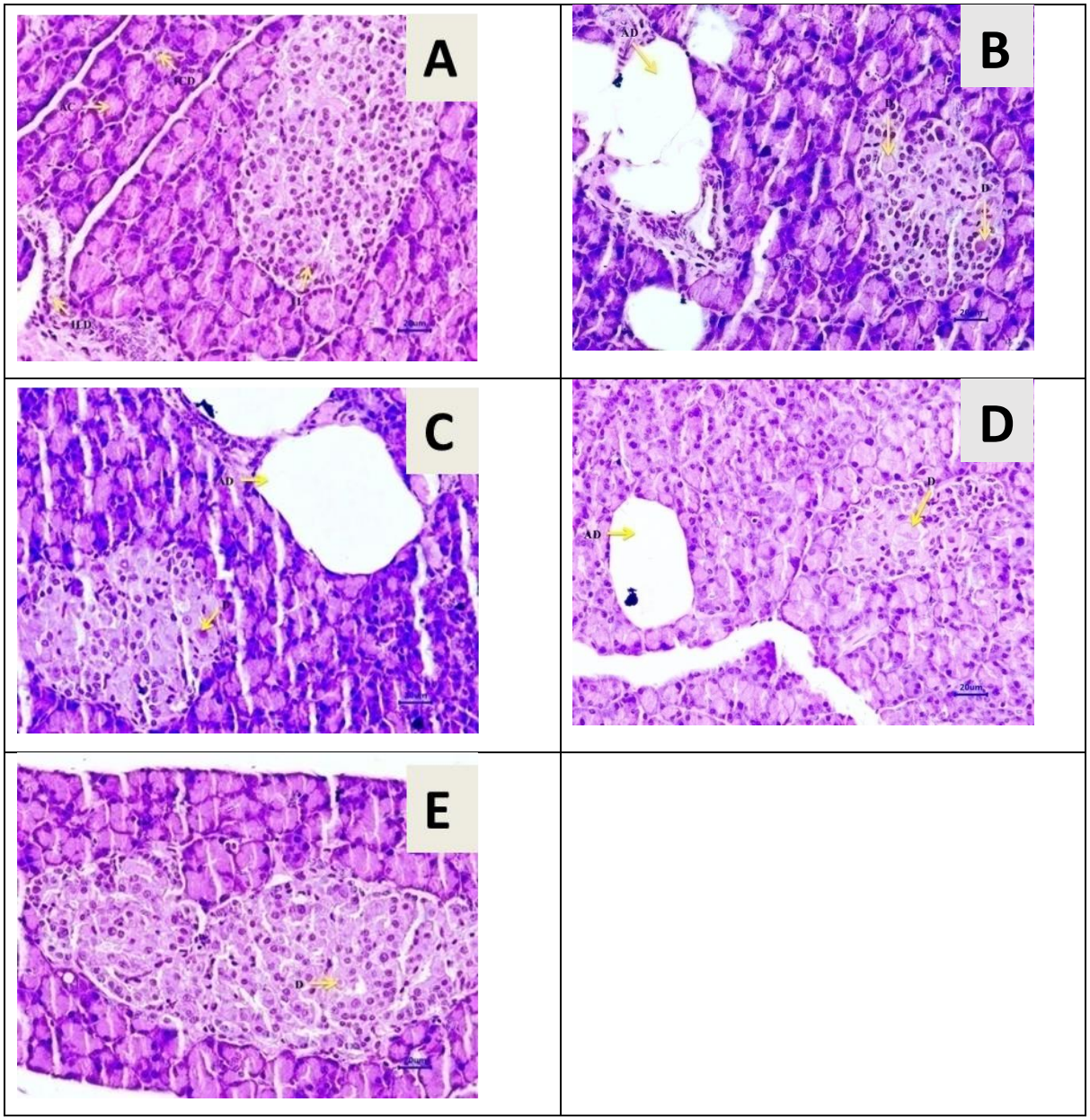



**Figure 17:** Effect of *T. chebula* on pancreatic tissue histology (H & E Stain, 400X).

**Effect of *T. chebula* aqueous extract treatment on SIRT1 expression**

Microscopic examination of the pancreas immunohistochemically stained slides revealed SIRT1 expression, which was revealed by positive brown particles in the islet of Langerhans and acini of the exocrine pancreas (Figure 12). Normal control pancreas showing marked expression of SIRT1 in the islet of Langerhans. Diabetic control pancreas showing mild expression of SIRT1 in Islet of Langerhans. *T. chebula* low dose pancreas showing mild expression of SIRT1 in the islet of Langerhans. *T. chebula* high dose pancreas showing moderate expression of SIRT1 in the islet of Langerhans. Standard drug pancreas showing

moderate expression of SIRT1 in the islet of Langerhans. Untreated diabetic animals showed a reduction in the optical density of immune-stained pancreatic tissue compared to normal animals showing a decrease in SIRT1 expression in pancreatic tissue. Treatment with 500 mg/kg of aqueous extract of *T. chebula* showed an increase in the optical density of immune-stained pancreatic tissue for SIRT1 expression in pancreatic tissue, but it was not a significant increment. At the same time, 1000 mg/kg of the extract showed a significant increase in SIRT1 expression in pancreatic tissue, indicated by an increase in the optical density of immune-stained pancreatic tissue for SIRT1 (Figure 13).

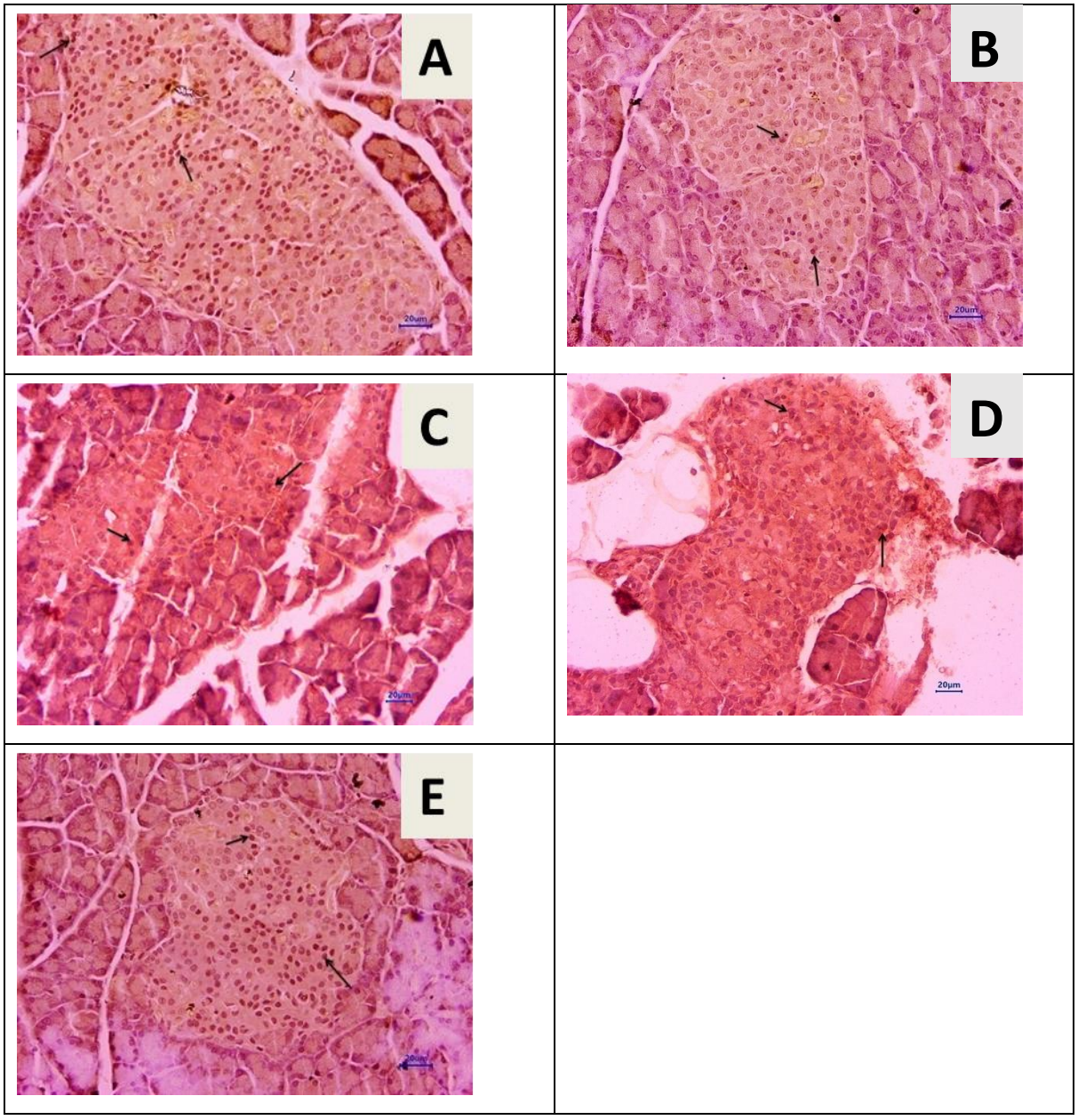


**Figure 18:** Immunohistochemical staining of Pancreas (400 X). Arrow showed expression of SIRT1 in the Islet of Langerhans.

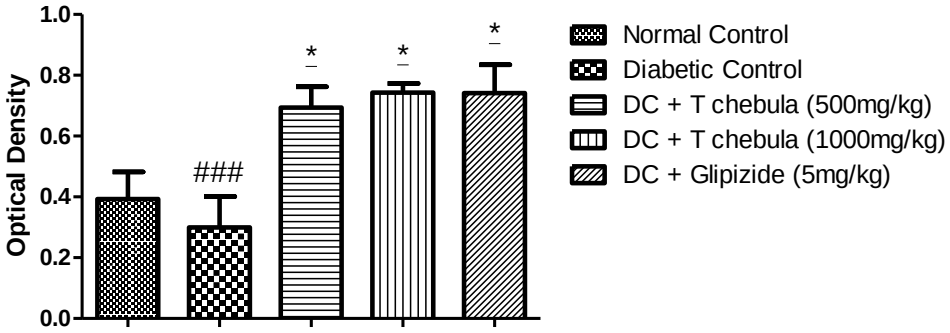



**Figure 19:** Change in Optical density for expression. All values are expressed as Mean ± S.E.M. (n=3). $^{*}p < 0.05$ when treatment groups were compared with disease control. $^{###}p < 0.001$ when the diabetic control group compared with the normal control group. (DC: Diabetic Control; *T chebula*: *Terminalia chebula*).

**Conclusion:**

The result of present study indicates that *Terminalia chebula* extract has significant antidiabetic effect.

**III Aim:** To study the effect of *Terminalia catappa* extract on the experimental model of Type 2 Diabetes Mellitus.

**Authentication of sample**: Botanical Survey of India (BSI), Pune

**Preparation of extract**: Double Maceration Technique

**Materials and methods**

**Plant Materials**

The dried fruits of T. catappa were procured from the SVKM's NMIMS University, Shirpur Campus, Dist.: Dhule, Maharashtra, India in November 2021. The collected plant leaves (5.0 Kg of leaves) after plugging from the tree were air dried under shade and ground into a powder using a mixture. The plant material was authenticated by Dr. D. L. Shirodkar, Botanist, Government of India; Ministry of Environment, Forests and Climate Change; Botanical Survey of India (BSI), Pune, Maharashtra, India. Plant species identification certificate number BSI / WRC / Iden. Cer. / 2021 / 2911210004749 has been deposited in the Department for future use.

**Chemicals**

Yeast extract and DL-methionine were obtained from Molychem, India. Cholesterol was obtained from Loba Chemie Pvt. Ltd., India. Casein was procured from Clarion casein limited, India. The lard was obtained from the local vendor. Streptozotocin was ordered from Sigma Aldrich (USA), and sodium chloride was purchased from SD Fine Chemicals, India. From Transasia Biomedicals Ltd, India, diagnostic kits were obtained. Glipizide was purchased from the local market.

**Preparation of Aqueous Extract**

Kernels of dried fruits of *T. catappa* were removed, powdered, and used for the preparation of aqueous extract by using the double maceration technique. Powdered fruits (500 g) were macerated with 5000 mL of distilled water for seven days. At the end of the seventh day, the extract was filtered through muslin cloth, and the filtrate was stored in the refrigerator till the subsequent processing. The marc was again kept for maceration with fresh distilled water for seven days. The extract was filtered through muslin cloth after seven days. The filtrate obtained in this step was mixed with the filtrate obtained in the first step. The extract was concentrated in a water bath (Make: Labline, Mumbai, India) and was stored in a refrigerator (Make: Toshiba, Model: UD-IND-SX, India) until further use (Kulkarni et al. 2013). The percentage yield of semisolid extract was found to be around 34%w/w.

**Quantification of ellagic acid and gallic acid in the aqueous extract by HPTLC method**

Quantification of ellagic acid and gallic acid from the aqueous extract of *T. catappa* was done by the High-Performance Thin Layer Chromatography (HPTLC) method. HPTLC analysis was carried out by using CAMAG HPTLC (Switzerland) equipped with visionCATS HPTLC Software, CAMAG Linomat V applicator, CAMAG Hamilton Micro syringe (100 µL), CAMAG TLC Scanner 4 (4.6 mm×25 cm, 5 µm), CAMAG UV Cabinet (254 & 366 nm), CAMAG Twin Trough Chambers (20 x 10 cm). The separation was achieved using a solvent system of *Methanol: Water: Formic acid (4:6:0.5) v/v/v,* and the stationary phase was pre-coated alumina backed, HPTLC Plate Silica gel 60 RP-18 (Merck). The scanning was done at 254 nm. Ellagic acid and gallic acid reference material were used as standards for quantifying tannins in the extract.

**Preparation of Standard Solutions**

The standard solutions of ellagic acid and gallic acid of the concentration of 100 µg/mL were prepared in methanol and applied to the HPTLC system.

**Experimental animals**

Male Sprague Dawley rats (160–170 g) were purchased from the National Institute of Biosciences, Pune, India. These animals were housed in the animal facility at a temperature and relative humidity of 22 ± 2°C and 75 ± 5%, respectively. The experimental animals were fed a basal regular pellet diet (Nutrimix Laboratory Animal Feed, MS, India) throughout the experiment. The high-fat diet consists of 25% protein, 58% fat, and 17% carbohydrate as a percentage of total kcal (Srinivasan et al. 2005). The high-fat diet composition per kilogram includes Normal pellet diet 365 g, Pork lard 310 g, Casein 250 g, Cholesterol 10 g, Vitamin D mineral mix 60 g, DL-Methionine 03 g, Yeast powder 01 g, Sodium Chloride 01 g. The Institutional Animal Ethics Committee approved the animal study protocol of Shri Vile Parle Kelavani Mandal. Considering the mortality during the study, number of animals per group were kept 10 so that minimum six observations can be obtained for evaluation.

**Induction of Diabetes**

Type 2 diabetes was induced using a low dose of Streptozotocin (STZ) (35 mg/kg, i.p., using 0.1 M cold citrate buffer at pH - 4.4, volume based upon body weight) (Rana, Singh, and Mehan 2018) (Kalaivani and Sankaranarayanan 2019) after two weeks of dietary modification using a

high-fat diet (Srinivasan et al. 2005). The experimental animals having blood glucose levels above 250 mg/dL were selected for the research study (Kulkarni and Garud 2016).

**Group I:** Normal control (received purified water)

**Group II:** Diabetic control rats (received purified water)

**Group III:** Diabetic rats were given *T. catappa* fruits aqueous extract (500 mg/kg body weight/day/rat) in distilled water.

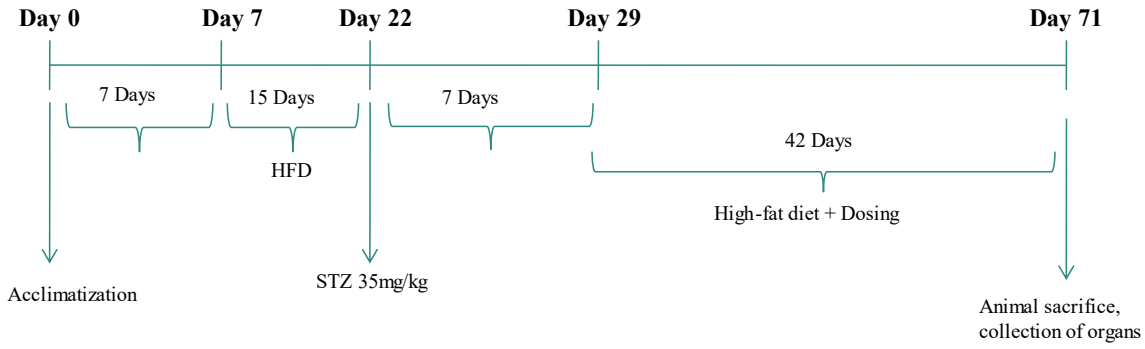
**Group IV:** Diabetic rats were given *T. catappa* fruits aqueous extract (1000 mg/kg body weight/day/rat) in distilled water.

**Group V:** Diabetic rats were administered with Glipizide (standard drug) (5 mg/kg body weight/day/rat) in 0.5% Sodium Carboxy Methyl Cellulose (Na CMC)

The treatment was given for six weeks by oral route with the help of oral gavage during the day time. All animals in animal house facility are supplied with purified water.

**Study Design:**

Animals received a single intraperitoneal injection of STZ at a dose of 35 mg/kg prepared in ice-cold citrate buffer (0.1 M, pH 4.5). Blood glucose was determined 7 days after STZ administration. Animals having blood glucose 250 mg/dL were considered as diabetic and selected for study.




**Pancreatic Tissue Collection and Processing**

In the end, animals from different treatment groups were humanely sacrificed, and the pancreas was isolated to determine oxidative stress parameters, histopathological studies, and immunohistochemistry.

**Evaluation of Parameters**

**Body weight**

The body weight of the animals from different groups was recorded at the end of the study.

**Estimation of Plasma Glucose and Lipids**

Blood samples of animals were collected, and plasma was separated. Plasma glucose level and lipid markers like total cholesterol, triglyceride, high-density lipoprotein, and low-density lipoprotein were performed by kits procured from Transasia Bio-Medicals Ltd., India, and evaluation was done using a Biochemical Analyzer, namely Erba Chem-7, Germany.

**Estimation of Liver Enzymes**

Alanine Transaminase and Aspartate Aminotransferase were measured per the protocol mentioned in the leaflets of the kits supplied by Transavia Bio-Medicals Ltd., India.

**Estimation of Glycohemoglobin Content**

Using the resin (ion exchange) method, as per the procedure mentioned in the kit supplied by Transasia Bio-Medicals Ltd., India, Glycohemoglobin content was determined.

**Estimation of Glucose Tolerance and Insulin Resistance**

**Estimation of Oral Glucose Tolerance Test (OGTT)**

An OGTT was performed as per the previously described method (Kulkarni and Garud 2016).

**Measurement of Plasma Insulin (PI), Insulin Sensitivity Index (ISI), and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR)**

Insulin level was estimated in plasma samples using rat insulin enzyme-linked immunosorbent assay (ELISA) kits supplied by Abbkine, USA.

Homeostatic Model Assessment- Insulin Resistance (HOMA-IR) and Insulin Sensitivity Index (ISI) were calculated using the following formula:

$$ISI = \ln (1 / FINS * FBG)$$

$$HOMA\text{-}IR = (Glucose \times Insulin) / 22.5$$

Where,

The concentration of insulin is expressed in mIU/L, Glucose in mmol/l

**Pancreatic Tissue Collection and Processing**

**Determination of Oxidative Stress Parameters**

Tissue homogenate (10% w/v) was prepared in phosphate buffer 50 mM (pH 7.4). Oxidative stress parameters like Malondialdehyde, Superoxide Dismutase, Catalase, and Reduced Glutathione were studied in pancreatic tissue homogenate. MDA and GSH levels were also estimated in pancreatic tissue homogenate. CAT was evaluated in the post-nuclear homogenate fraction, whereas SOD was assessed in the post-mitochondrial fraction of homogenate (Ellman, 1959; Ohkawa, Ohishi, and Yagi, 1979; Paoletti, Mocali and Aldinucci, 1990) (Garud and Kulkarni 2017).

**Histopathology of Pancreatic Tissue**

The study was processed as reported earlier (Kulkarni and Garud 2016) (Oza and Kulkarni 2018). The stained slides were studied under a motic microscope for histopathological changes.

**Immunohistochemistry**

As reported earlier, the pancreatic tissue immunohistochemical staining was carried out to express SIRT1 (Oza and Kulkarni 2018).

## Statistical analysis

The estimation was done using software, namely, GraphPad Prism for Windows ver. 5.00. The data are expressed in Mean ± SEM. Significant differences between the experimental animal groups were assessed by one-way analysis of variance test (ANOVA) followed by Bonferroni's multiple comparison tests to determine the significance level. *p*-value less than 0.05 was considered significant.

**Results:**

**Quantitative estimation of Ellagic acid and Gallic acid in the aqueous extract of fruits of *Terminalia catappa* by HPTLC**

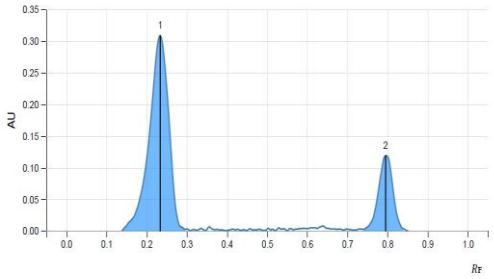



**Figure 5:** Chromatogram of Standard Ellagic acid (1) and Gallic acid (2)

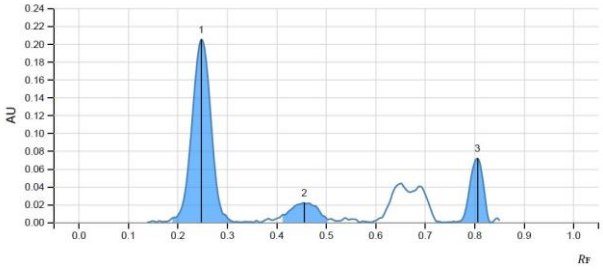


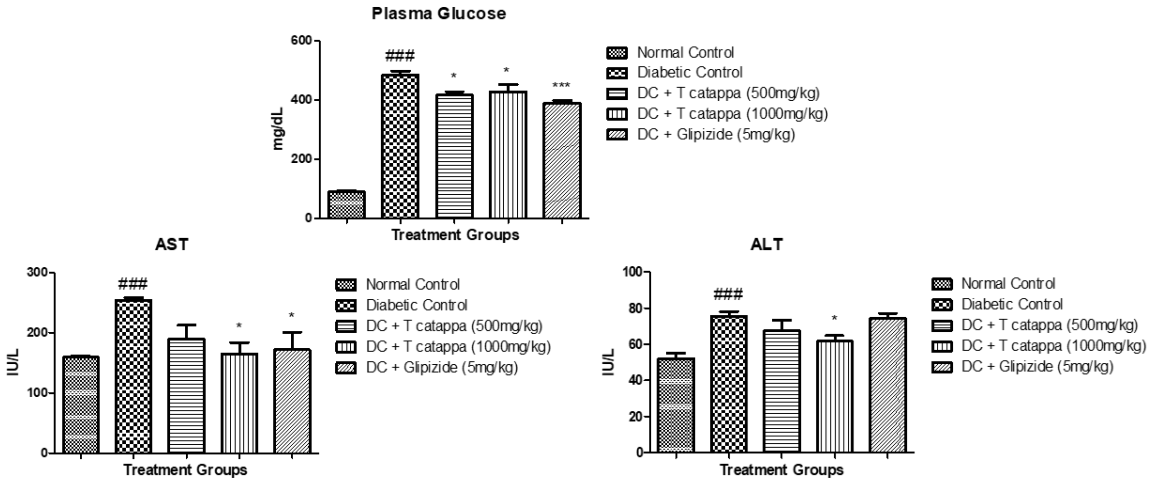

**Figure 6:** Chromatogram of Aqueous extract of fruits of *Terminalia catappa* showing the presence of Ellagic acid (1) and Gallic acid (3)

**The retention factor** for standard Ellagic acid and Gallic acid was obtained at 0.234 and 0.795, respectively.

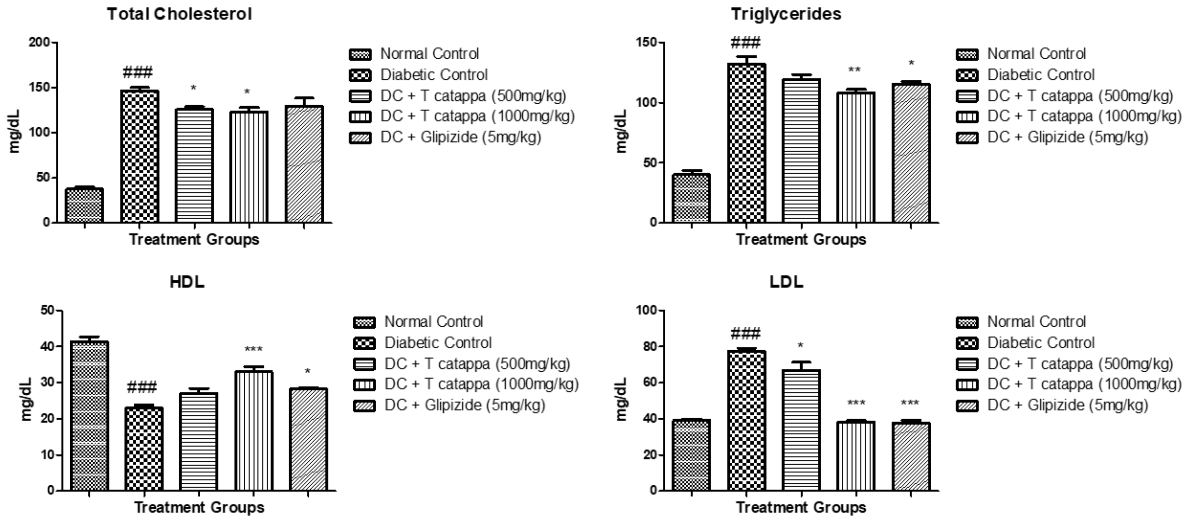
**Mobile Phase:** Methanol: Water: Formic Acid (4:6:0.5) v/v/v

| Actives | Standards | | Tannins in Extract | | % Contents of actives in extracts |
|---|---|---|---|---|---|
| | Concentration (µg/mL) | Peak area | Peak area | Concentration (µl) | |
| Ellagic acid | 100 | 0.01724 | 0.01032 | 8 | 0.0015 |
| Gallic acid | 100 | 0.00498 | 0.00167 | 8 | 0.0062 |

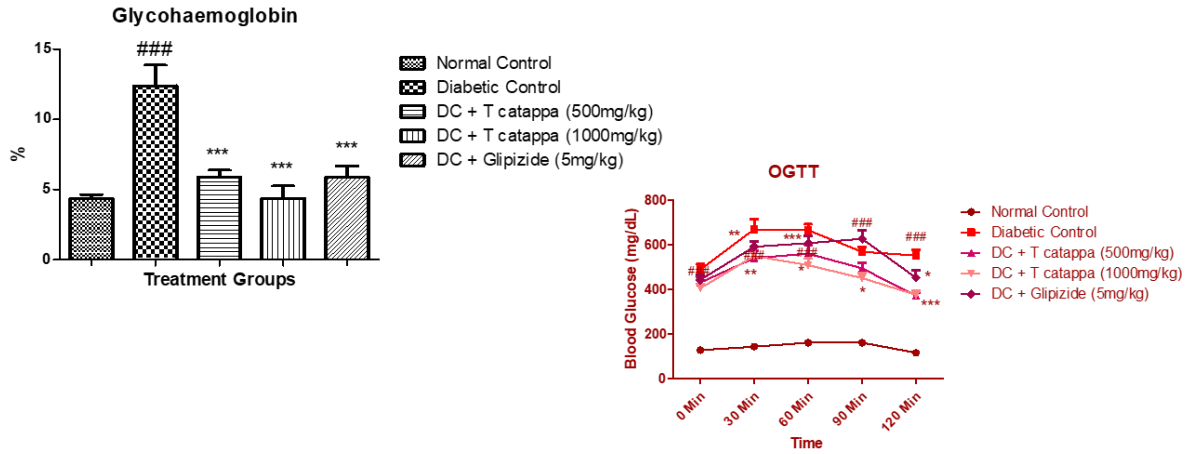
## Plasma Glucose and Liver Enzymes



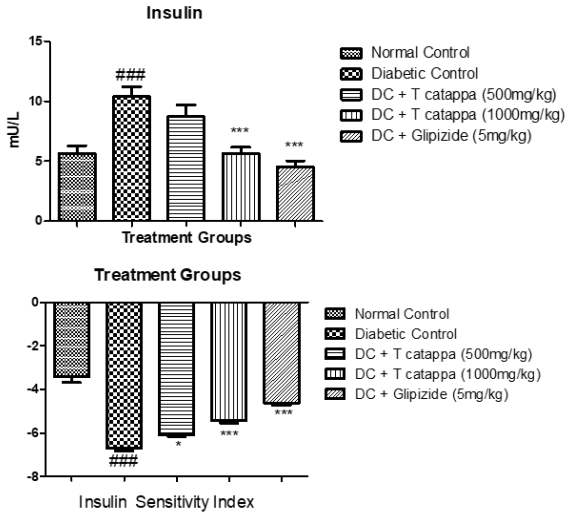
## Lipid Parameters:

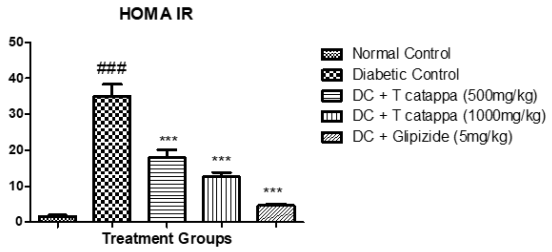



## Glycohemoglobin and Oral Glucose Tolerance Test:




## Measurement of Plasma Insulin (PI), Insulin Sensitivity Index (ISI) and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR):

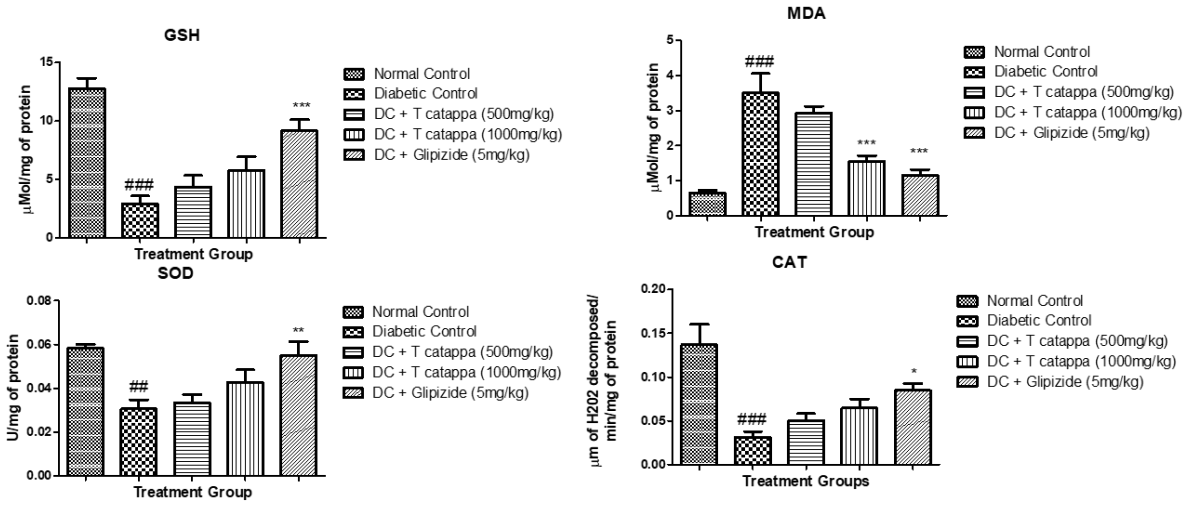



All values are expressed as Mean ± S.E.M. (n=7). *p< 0.05, **p<0.01 and ***p < 0.001, when treatment group compared with diabetic control. ### p < 0.001 when the diabetic group compared to the normal control group.

ISI= Insulin Sensitivity Index
HOMA IR = Homeostatic Model Assessment of Insulin Resistance

## Oxidative Stress Parameters:




## Conclusion:

The result of present study indicates that *Terminalia catappa* extract has significant antidiabetic effect.

## 7. Publications and Presentations:

| No | Details | Journal | IF | Status |
|---|---|---|---|---|
| **1** | **Ojaskumar D. Agrawal**, Yogesh A. Kulkarni 'Mini-Review of Analytical Methods used in Quantification of Ellagic Acid' *Reviews in Analytical Chemistry De Gruyter* 2020; 39: 31–44. | Reviews in Analytical Chemistry | 2.174 | Published |
| **2** | **Ojaskumar D. Agrawal**, Yogesh A. Kulkarni Treatment with *Terminalia chebula* Extract Reduces Insulin Resistance, Hyperglycemia and improves SIRT1 Expression in Type 2 Diabetic Rats. | Life | 3.253 | Under Review |
| **3** | **Ojaskumar D. Agrawal**, Yogesh A. Kulkarni Aqueous Extract of *Terminalia bellirica* Fruits Improves Insulin Sensitivity, Controls Hyperglycemia and Improves SIRT1 Expression in High Fat Diet and Streptozotocin-Induced Type 2 Diabetes in Rats. | Tropical Medicine and Infectious Disease | 3.711 | Communicated |
| **4** | **Ojaskumar D. Agrawal**, Yogesh A. Kulkarni Improvement in insulin sensitivity and controls hyperglycemia in type 2 diabetes by *Terminalia catappa* fruit extract. | Chemistry and Biodiversity | 2.40 | Ready to Communicate |

**Book Chapters:**

| No | Title of Book | Title of the Book Chapter | Authors | Publisher | Status |
|---|---|---|---|---|---|
| **1** | '*Phytotherapy in the Management of Diabetes and Hypertension'* Volume 3, Editor: Mohamed Eddouks | Management of Diabetes Mellitus by Natural Products: Glucagon-like Peptide 1 Perspective, 2020, Vol. 3, 95-126. | Ojaskumar D. Agrawal and Yogesh A. Kulkarni | Bentham Science | Published |
| **2** | Chemistry Inside Spices and Herbs: Research and Developments | Phytochemistry and Pharmacology of *Terminalia chebula*: an Update Volume II; (2022) 2: 229-260 | Ojaskumar D. Agrawal, Yogesh A. Kulkarni | Bentham Science | Published |
| **3** | Diet and Nutrition in Neurosciences | Dietary Neurotoxins: An Overview | Ojaskumar D. Agrawal, Yogesh A. Kulkarni | Elsevier | In Press |

**Presentations:**

| No | Authors | Title | Name of Conference | Venue | Year |
|---|---|---|---|---|---|
| **1** | Agrawal O.D., Kulkarni Y.A. | Peril and Promise of Stem Cells in Psoriasis | The Annual Conference on Pharmacological Society – *IPSCON 2017* | SVKM's NMIMS, Mumbai | 15th - 17th February 2018 |
| **2** | Agrawal O.D., Kulkarni Y.A. | Sodium Chloride and Diabetic Complications | International Diabetic Federation (IDF), Diabetes Complications Congress | Hyderabad, India | 25th - 27th October 2018 |
| **3** | Agrawal O.D., Kulkarni Y.A. | Phytochemistry and Pharmacology of *Terminalia chebula*: An Update | 5th IUPHAR World Conference on The Pharmacology of Natural Products | ICMR – NIN Hyderabad | 05th – 07th December 2019 |
| **4** | ***Ojaskumar D. Agrawal, Yogesh A. Kulkarni** | **Effect of *Terminalia chebula* extract in Type 2 Diabetic Rats** | **9th International Congress of the Society for Ethnopharmacology, India (SFEC 2022)** | **JSS COP, Mysuru, India** | **22nd - 24th April 2022** |

***Received the Best Poster Presentation Award**

## 8. Conclusion of Project work

The result of present study indicates that *Terminalia bellirica, Terminalia chebula and Terminalia catappa* extract has significant antidiabetic effect.

## 9. References


Banu, Sofia, and Ananya Bhowmick. 2017. "Therapeutic Targets of Type 2 Diabetes: An Overview." *MOJ Drug Design Development & Therapy* 1(3): 1–6. http://medcraveonline.com/MOJDDT/MOJDDT-01-00011.php.

Chang, CLT et al. 2013. "Herbal Therapies for Type 2 Diabetes Mellitus: Chemistry, Biology, and Pote...: EBSCOhost." *Evidence-Based Complementary and Alternative Medicine* 2013: 1–33. http://web.b.ebscohost.com.ezproxy.think.edu.au/ehost/pdfviewer/pdfviewer?sid=c049c6b6-7b8f-4672-8838-059fb794dac7@sessionmgr120&vid=1&hid=118.

ELLMAN, G L. 1959. "Tissue Sulfhydryl Groups." *Archives of biochemistry and biophysics* 82(1): 70–77. https://linkinghub.elsevier.com/retrieve/pii/0003986159900906.

Garud, Mayuresh Sudamrao, and Yogesh Anant Kulkarni. 2017. "Eugenol Ameliorates Renal Damage in Streptozotocin-Induced Diabetic Rats." *Flavour and Fragrance Journal* 32(1): 54–62.

Kalaivani, Karunanithi, and Chandrasekaran Sankaranarayanan. 2019. "Isopulegol Ameliorates Dyslipidemia by Modulating Adipokine Secretion in High Fat Diet / Streptozotocin Induced Diabetic Rats." *Journal of Drug Delivery and Therapeutics* 9(4-A): 126–36. http://www.jddtonline.info/index.php/jddt/article/view/3429 (December 27, 2022).

Kulkarni, Yogesh A. et al. 2013. "Effect of Gmelina Arborea Roxb in Experimentally Induced Infl Ammation and Nociception." *Journal of Ayurveda and Integrative Medicine* 4(3): 152–57. /pmc/articles/PMC3821189/ (August 9, 2022).

Kulkarni, Yogesh A., and Mayuresh S. Garud. 2016. "Bauhinia Variegata (Caesalpiniaceae) Leaf Extract: An Effective Treatment Option in Type I and Type II Diabetes." *Biomedicine & Pharmacotherapy* 83: 122–29. http://www.ncbi.nlm.nih.gov/pubmed/27470559 (June 8, 2021).

Ohkawa, Hiroshi, Nobuko Ohishi, and Kunio Yagi. 1979. "Assay for Lipid Peroxides in Animal Tissues by Thiobarbituric Acid Reaction." *Analytical Biochemistry* 95(2): 351–58.

Oza, Manisha J., and Yogesh A. Kulkarni. 2018. "Formononetin Treatment in Type 2 Diabetic Rats Reduces Insulin Resistance and Hyperglycemia." *Frontiers in Pharmacology* 9(JUL): 1–11. https://www.frontiersin.org/article/10.3389/fphar.2018.00739/full.

Ozougwu, Ozougwu. 2013. "The Pathogenesis and Pathophysiology of Type 1 and Type 2 Diabetes Mellitus." *Journal of Physiology and Pathophysiology* 4(4): 46–57. http://academicjournals.org/journal/JPAP/article-abstract/974E7B515872.

Paoletti, Francesco, Alessandra Mocali, and Donatella Aldinucci. 1990. "Superoxide-Driven NAD(P)H Oxidation Induced by EDTA-Manganese Complex and Mercaptoethanol." *Chemico-Biological Interactions* 76(1): 3–18.

Rana, Surbhi, Lakhwinder Singh, and Sidharth Mehan. 2018. "Forskolin, Ameliorates Mitochondrial Dysfunction in Streptozotocin Induced Diabetic Nephropathy in Rats." *Asian Journal of Pharmacy and Pharmacology* 5(1): 199–206. http://ajpp.in/uploaded/p258.pdf.

Safavi, Maliheh, Alireza Foroumadi, and Mohammad Abdollahi. 2013. “The Importance of Synthetic Drugs for Type 2 Diabetes Drug Discovery.” *Expert Opinion on Drug Discovery* 8(11): 1339–63. http://www.tandfonline.com/doi/full/10.1517/17460441.2013.837883.

Sekar, N. et al. 1990. “Insulinic Actions of Vanadate in Diabetic Rats.” *Pharmacological Research* 22(2): 207–17.

Srinivasan, K. et al. 2005. “Combination of High-Fat Diet-Fed and Low-Dose Streptozotocin-Treated Rat: A Model for Type 2 Diabetes and Pharmacological Screening.” *Pharmacological research* 52(4): 313–20. https://linkinghub.elsevier.com/retrieve/pii/S1043661805001039 (June 8, 2021).

Vaz, Julius a, and Ashis Patnaik. 2012. “Diabetes Mellitus: Exploring the Challenges in the Drug Development Process.” *Perspectives in clinical research* 3(3): 109–12. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3487225&tool=pmcentrez&rendertype=abstract.

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